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THE EFFECT OF ANDROGENIC HORMONES UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE INJECTIONS AND UPON THE ANTI-INFLAMMATORY ACTION OF CORTISONE[1]

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ZIZINE, Simpson and Evans (1950) reported that testosterone brought about a significant degree of adrenal repair in hypophysectomized animals. Since cortisone produces adrenal atrophy which resembles in many respects that seen after hypophysectomy, it was of interest to determine whether androgenic hormones would antagonize this effect of cortisone.

MATERIAL AND METHODS

Young adult male Holtzman rats were used in this study, except for the experiments where hypophysectomized rats were employed; the latter were purchased from the Hormone Assay Laboratories, Inc., Chicago. Steroids were injected in saline suspension subcutaneously, once daily. Some experiments were performed with testosterone propionate, but the androgen employed in most of the study was 17(α)-methyl-Δ^5-androstene-3(β)-17(β)-diol (methyl-androstenediol or mestenediol)* (M.A.D.). The cytochemical methods used in this investigation have been described elsewhere (Stebbins, 1951a).

RESULTS

Effect of testosterone in cortisone-treated rats. Table 1 gives the results of experiments in which cortisone was injected daily for a period of 9 days. Although adrenal atroply is not maximal within that period of time, it

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* The authors gratefully acknowledge generous supplies of methylandrostenediol through the courtesy of Dr. K. W. Thompson, Organon, Inc., Orange, N. J.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 9 DAYS, MEAN VALUES FOR GROUPS OF 8 RATS

Treatment	Body weight		Thymus, mg.	Adrenals	
	Initial, g.	Final, g.		mg.	Mg./100 g. body wt.
Cortisone acetate 1.5 mg.	107	114	65	14.5	12.8
Cortisone acetate 3 mg.	98	104	24	14.1	13.5
Cortisone acetate 1.5 mg.+testosterone propionate 2.5 mg.	101	119	26	19.2	16.3
Cortisone acetate 3 mg.+testosterone propionate 2.5 mg.	109	108	18	19.0	17.8
Untreated controls	109	165	522	29.9	18.1

has been shown that most of the atrophy produced by cortisone occurs within the first 10 days (Winter, Silber and Stoerk, 1950). In the present experiment, there was not much difference in the degree of adrenal atrophy produced in 9 days by 1.5 mg. and by 3 mg. of cortisone acetate. Testosterone propionate, when given in conjunction with cortisone, prevented the adrenal atrophy to a considerable extent. It did not, however, prevent the suppression of body growth or atrophy of the thymus which is characteristic of cortisone-treated rats.

Effect of methylandrostenediol in cortisone-treated rats. When M.A.D. was given in graded doses in combination with 2.5 mgm. daily of cortisone acetate, a degree of prevention of adrenal atrophy was obtained which was directly related to the dose of M.A.D. employed up to 0.5 mgm. daily (Table 2). 0.05 mg. of M.A.D. produced no noticeable effect, but when 0.5 mg. was given to the cortisone-treated rats, the adrenals remained with-

TABLE 2. EFFECT OF GRADED DOSES OF METHYLANDROSTENEDIOL (M.A.D.) UPON THE ADRENAL ATROPHY PRODUCED BY CORTISONE. TREATMENTS CONTINUED FOR 19 DAYS (EXPERIMENT 1) OR 18 DAYS (EXPERIMENT 2); MEAN VALUES FOR GROUPS OF 9 TO 10 RATS

Treatment	Experiment No.	Body weight		Thymus, mg.	Adrenals	
		Initial, g.	Final, g.		Mg.	mg./100 g. body wt.
Cortisone acetate 2.5 mg.	1	106	92	23	13.0	14.3±0.66*
Cortisone acetate 2.5 mg.	2	120	111	23	12.9	11.8±0.59
Cortisone acetate 2.5 mg. +M.A.D. 0.05 mg.	1	113	106	22	14.7	13.9±0.60
Cortisone acetate 2.5 mg. +M.A.D. 0.1 mg.	1	106	99	20	15.0	15.1±0.68
Cortisone acetate 2.5 mg. +M.A.D. 0.25 mg.	1	111	98	20	15.8	16.2±0.89
Cortisone acetate 2.5 mg. +M.A.D. 0.5 mg.	2	115	116	25	23.7	20.5±1.45
Methylandrostenediol 0.5 mg.	2	125	188	381	43.8	24.2±1.83
Uninjected controls	1	111	190	435	39.3	21.3±1.81
Uninjected controls	2	125	198	440	42.7	21.8±1.07

* Standard error.

in the limits of normal size for rats of corresponding body weights. Higher doses of M.A.D. (not shown in the table) produced no greater effect upon adrenal weight. Thymus atrophy was virtually complete in all the animals treated with cortisone regardless of whether or not M.A.D. was also administered. The M.A.D. did not counteract the suppression of body growth by cortisone. M.A.D. by itself, in a dose of 0.5 mg. daily for 18 days produced about a 13 per cent decrease in thymus weight, but body weight and adrenal size were not significantly affected.

Histological examination of the adrenal glands of these animals demonstrated that the changes produced by cortisone could be largely prevented by concurrent administration of M.A.D. Cortisone produced a decrease in width of the cortex due to diminution in size of the cells of the zonae fasciculatis and reticularis (Fig. 1). Cytoplasmic lipid (Fig. 7) and birefringent material (Fig. 11) were also largely lost. The glomerulosa remained largely unchanged except for a possible slight widening, and the presence of coarse birefringent particles. M.A.D. in doses of 0.5 mgm. daily prevented these changes (Figs. 2, 8, and 12), and the adrenals of the animals presented a microscopic picture similar to that of uninjected controls. No microscopic changes were observed in the adrenals of rats treated with M.A.D. alone.

TABLE 3. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL UPON ADRENAL WEIGHT IN HYPOPHYSECTOMIZED RATS

Treatment	Mean body weight, g.		Mean adrenal weight
	Initial	Final	Mg. ±S.E.
I. Cortisone 2 mg. daily	175	125	13.79 ± .50
II. M.A.D. 3 mg. daily	180	168	15.50 ± .83
III. Cortisone + M.A.D.	177	136	20.85 ± .94
IV. Uninjected controls	174	160	15.44 ± .75

Effect of cortisone and methylandrostenediol upon hypophysectomized rats. Rats that were hypophysectomized about 4 days were divided into groups as shown in Table 3. This experiment was performed twice, but as the results were identical in the two experiments, the data have been combined in the table. Each figure in Table 3 represents the mean of 16 to 20 rats. Treatment was continued for 18 days. The dose of M.A.D. was higher than that found in the preceding experiment to be necessary for optimal effects. Indeed, probably not all the administered androgen was absorbed, as deposits were found at injection sites when the rats were sacrificed. However, sufficient M.A.D. was absorbed to have a pronounced effect in combination with the cortisone.

None of the treatments succeeded in preventing the loss in body weight following hypophysectomy. Indeed, those treated with cortisone lost significantly ($P < 0.01$) more than did the untreated controls and treat-

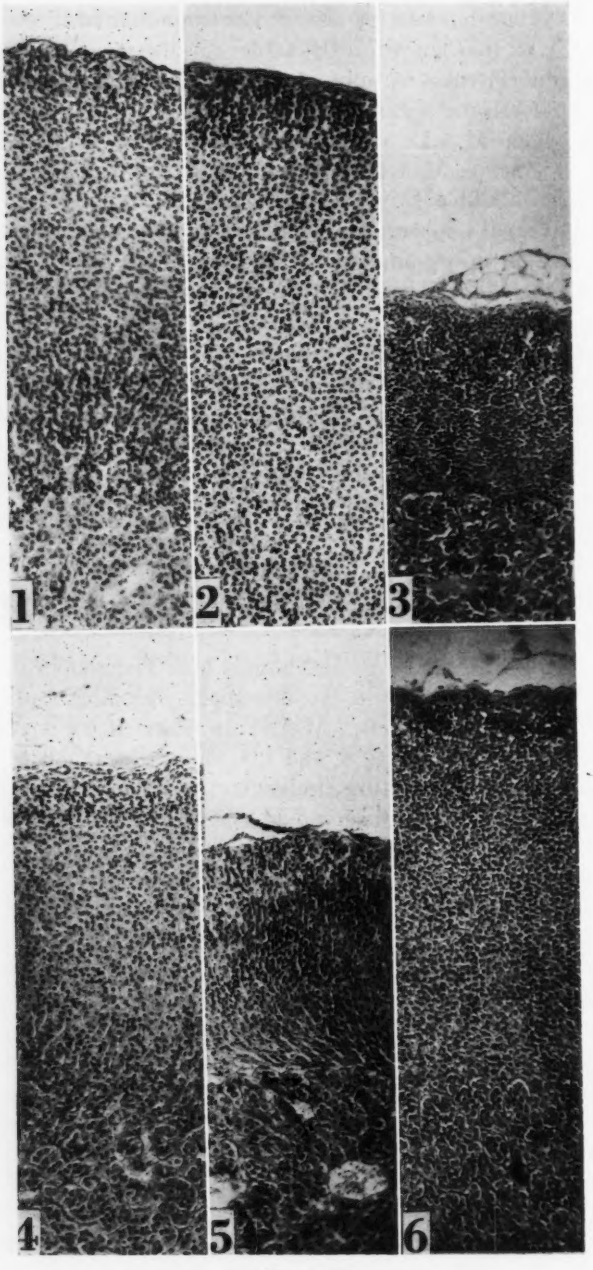

PLATE I

ment with the androgen did not prevent this excessive weight loss (P for difference in body weight loss, Groups I and III >0.05). M.A.D. alone did not significantly alter the weight loss in comparison with uninjected controls ($P>0.05$). Therefore, only the cortisone affected weight loss in the hypophysectomized rats; this loss in weight was greater than that commonly seen in intact rats treated with a similar dose of cortisone.

Effects on adrenal weights were very different from effects on body weight. Neither cortisone nor M.A.D. restored the adrenals of the hypophysectomized rats, but the combination of the two steroids restored the adrenals almost to the size of those observed in intact rats of comparable body weights.

The atrophy of the adrenals in the hypophysectomized rats receiving cortisone is shown in Figure 3, while Figure 4 demonstrates the repair occurring as a result of combination therapy of the two steroids. The atrophy in the presence of cortisone alone was not significantly greater than that seen in uninjected hypophysectomized controls, in confirmation of Stebbins (1951b). The extremely small size of the cells of the inner zones of the cortex of these atrophic glands (Fig. 3) was due to loss of cytoplasm, particularly of cytoplasmic lipid; the glomerulosa, in contrast, was thickened and showed a heavy deposition of lipid (Fig. 9). M.A.D. alone did not effectively restore the normal histological picture. The cells of the inner zones in these rats contained large vacuoles in which no lipid could be demonstrated. Considerable repair of the adrenal cortex could be seen in

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PLATE I. PARAFFIN SECTIONS OF RAT ADRENALS ×108. HEMATOXYLIN-EOSIN

FIG. 1. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. The cells of the inner cortical zones are reduced in size due to loss of cytoplasmic lipid. The glomerulosa appears slightly wider than normal.

FIG. 2. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. methylandrostenediol for 18 days. Inner cortical cells contain more cytoplasmic lipid than in Fig. 1. The glomerular zone is essentially normal.

FIG. 3. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. Atrophic cells of inner cortex have lost lipid and nuclei are small and closely packed. Glomerulosa appears thickened.

FIG. 4. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Cells of zonae fasciculariss and reticularis are larger and contain more cytoplasmic lipid and the glomerulosa is thinner than in Fig. 3.

FIG. 5. Intact rat injected daily with 2.5 mg. of cortisone acetate for 32 days. The section gives evidence of severe contraction of inner cortex with virtual extinction of cellular lipid and small closely arranged nuclei. Glomerulosa appears thickened.

FIG. 6. Intact rat injected with 2.5 mg. of cortisone acetate for 31 days and 0.5 mg. of methylandrostenediol daily from 18th to 31st day. Section displays a much wider inner cortex due to presence of lipid and pink-staining cytoplasm. Subglomerular and outer fascicular cells appear more closely packed than cells deeper in cortex.

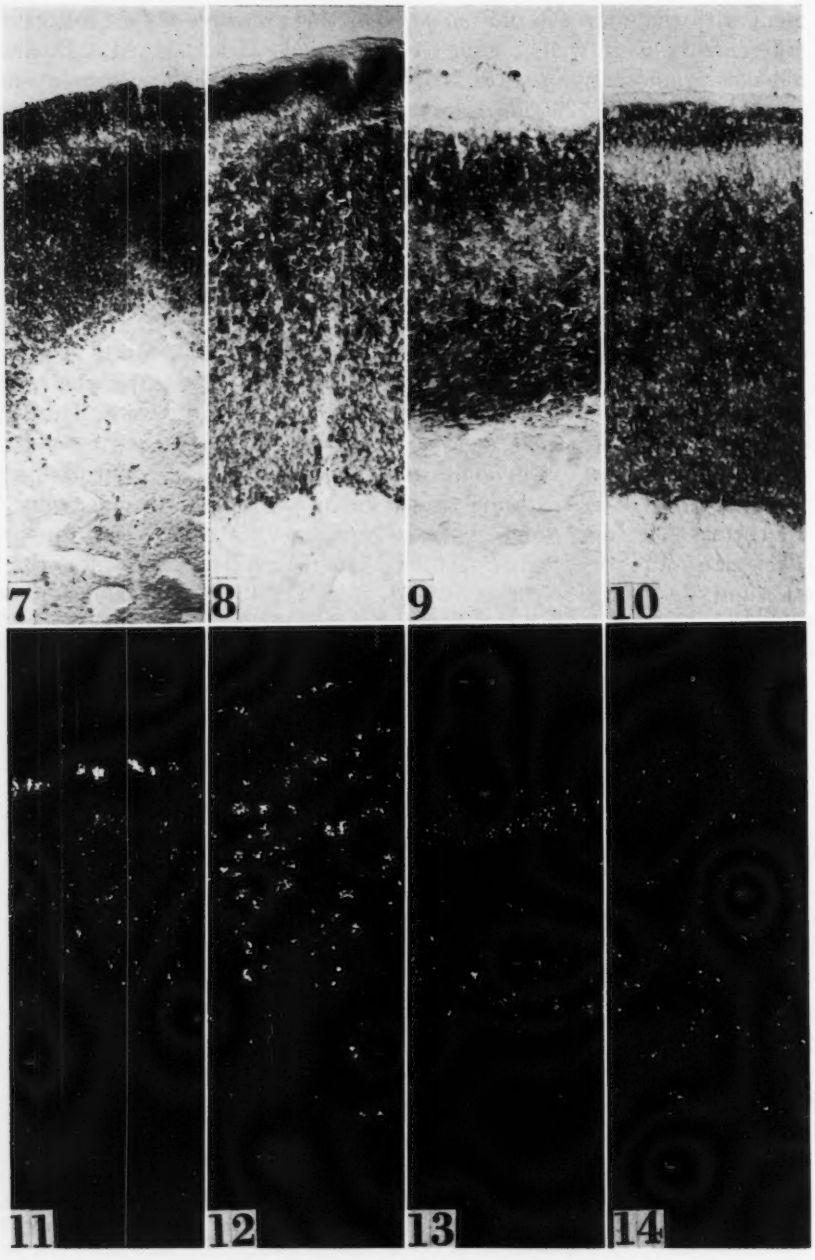

PLATE II

the animals receiving combined therapy with the two steroids (Fig. 4). The adrenal cortices of these animals were much wider than those of untreated controls, or of those receiving either steroid alone. The cells were larger and more distinct and contained considerably more lipid (Fig. 10), while the birefringent particles were finer and more diffusely arrayed (cf. Figs. 13 and 14). In these respects the glands more nearly resembled those of intact rats. In general, the histological study confirmed the data obtained on adrenal weights.

Reversal of cortisone-induced adrenal atrophy. Having determined that M.A.D. could prevent cortisone-induced atrophy, and that the combination of steroids was effective in hypophysectomized rats, we next attempted to determine whether M.A.D. could restore the adrenals of intact rats in which atrophy had already been produced by cortisone, and also whether M.A.D. could hasten recovery of the adrenals after discontinuance of cortisone injections. Accordingly, 78 male rats weighing about 95 grams were injected with 2.5 mg. of cortisone daily for 17 days. These animals were then divided into groups of six rats, as shown in Table 4. In some groups (Ia to Id) cortisone was continued for varying lengths of time to obtain control figures on the degree of atrophy produced by this steroid.

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PLATE II. DISTRIBUTION OF LIPID AND BIREFRINGENT MATERIAL IN FROZEN SECTIONS OF RAT ADRENALS ×108. SUDAN BLACK B.

FIG. 7. Adrenal of intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Shrunken cells of inner cortex have lost appreciable amount of lipid.

FIG. 8. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Larger cortical cells are laden with lipid.

FIG. 9. Hypophysectomized rat treated with 2.5 mg. of cortisone acetate for 18 days. Lipid is lost from outer area of inner cortex. Thickened glomerulosa contains large amount of cellular lipid.

FIG. 10. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Large cells of fasciculata and reticularis are laden with lipid. Thinner glomerulosa shows less lipid than in Figure 9.

FIG. 11. Intact rat injected daily with 2.5 mg. of cortisone acetate for 18 days. Coarse birefringent accumulations appear in glomerulosa with very fine particles in inner cortex. Subglomerular zone between them is empty.

FIG. 12. Intact rat injected daily with 2.5 mg. of cortisone acetate and 0.5 mg. of of methylandrostenediol. Fine birefringent particles appear in the glomerulosa. A mixture of fine and coarse particles are seen in the fasciculata and reticularis.

FIG. 13. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate for 18 days. The glomerulosa displays closely arranged fine particles. The atrophic cells of inner cortex contain for the most part very fine delicate birefringent particles.

FIG. 14. Hypophysectomized rat treated daily with 2.5 mg. of cortisone acetate and 0.5 mg. of methylandrostenediol for 18 days. Very fine particles of glomerulosa are more freely distributed than in Figure 13. Larger cells of inner cortex contain larger and more distinct birefringent material.

TABLE 4. REVERSAL BY METHYLANDROSTENEDIOL (M.A.D.) OF ADRENAL ATROPHY PRODUCED BY CORTISONE ACETATE

Treatment	Mean adrenal weight, mg.
I. Cortisone acetate 2.5 mg. daily for:	
a. 17 da.	11.2
b. 21 da.	11.5
c. 24 da.	11.7
d. 31 da.	10.6
II. Cortisone acetate 2.5 mg. daily for:	
a. 21 da., M.A.D. 0.5 mg. last 4 da.	15.0
b. 24 da., M.A.D. 0.5 mg. last 7 da.	14.2
c. 31 da., M.A.D. 0.5 mg. last 14 da.	15.4
III. Cortisone acetate 2.5 mg. for 17 da.:	
a. sacrificed 4 da. after last injection	16.3
b. sacrificed 7 da. after last injection	23.7
c. sacrificed 14 da. after last injection	27.7
IV. Cortisone acetate 2.5 mg. for 17 da., then cortisone discontinued and M.A.D. 0.5 mg. started:	
a. sacrificed 4 da. after last cortisone	17.6
b. sacrificed 7 da. after last cortisone	21.6
c. sacrificed 14 da. after last cortisone	25.4

In comparable groups (IIa to IIc) M.A.D. was given in conjunction with cortisone during the last portion of the period of cortisone treatment. Additional groups were studied to determine the rate of adrenal recovery after discontinuance of cortisone without (IIIa to IIIc) and with (IVa to IVc) M.A.D.

The data demonstrate that after cortisone had been injected for 17 days, no further adrenal atrophy took place when injections were prolonged (Groups Ia to Id). When adrenal atrophy was allowed to occur, and then M.A.D. was given along with continuing cortisone injections (Groups IIa to IIc), a significant degree of recovery occurred ($P = 0.02$). This recovery did not completely restore the adrenals to normal size, however. After cortisone was discontinued, recovery of the adrenals took place at the same rate whether or not M.A.D. was administered during the recovery period (Groups III and IV); in each case, the difference between adrenal size in the "recovery" animals with and without M.A. D. was not significant ($P > 0.05$).

The histological picture of adrenal repair shown in Figure 6 is taken from an animal of Group IIc. This is in contrast to the markedly atrophic adrenals of the animals which received cortisone for the same length of time without M.A.D. (Fig. 5, animal of Group Id). Recovery of the adrenal cortex after discontinuance of cortisone has been described in a previous communication (Winter, Silber and Stoerk, 1950). Essentially, the same restoration of the histological picture was seen in the present series. Histologically, there was not much difference between the recovery with and without M.A.D., although there seemed to be a slight difference in favor of the M.A.D.-treated rats after 4 days of recovery

TABLE 5. EFFECT OF CORTISONE ACETATE AND METHYLANDROSTENEDIOL (M.A.D.) UPON THE EDEMA PRODUCED BY INJECTION OF IRRITANT (0.1 CC. OF 0.5 PER CENT $AgNO_3$ SOLUTION IN RAT'S FOOT)

Treatment	Amount of edema*	
	3 hours	24 hours
Untreated controls	51.8	42.2
M.A.D.—3 mg. daily for 3 da.	42.5	48.7
Cortisone—1.5 mg. daily for 3 da.	33.8	30.8
M.A.D.—3 mg. +cort. ac. 1.5 mg. daily for 3 da.	25.0	27.2

* "Amount of edema" = difference in cross-sectional area of foot before and after irritant (see text).

(Group IVa compared to IIIa) and a slight difference in favor of those without M.A.D. in the later recovery groups (IVb and c compared to IIb and c). These differences were too small to be certain of their significance.

Effect of methylandrostenediol upon the anti-inflammatory action of cortisone. Since M.A.D. clearly antagonized the production of adrenal atrophy by cortisone, it was of interest to determine whether this androgen would also antagonize the anti-inflammatory effects of cortisone. Sely (1949) has shown that cortisone inhibits the local effect of irritants injected under the plantar surface of the rats' foot. We have employed a similar technique, using as an irritant 0.1 cc. of 0.5 per cent $AgNO_3$ solution, and measuring the edema 3 hours and 24 hours after the irritant. The animals were pretreated for 3 days with steroids subcutaneously. The results are given in Table 5. The "amount of edema" was calculated as follows: measurements of two diameters of the rat's foot were made by calipers at about the mid-metacarpal line. Assuming the cross-section of the foot to be an ellipse, the cross-sectional area in sq. mm. was calculated. The difference between this area before and after injection of irritant was termed "amount of edema."

TABLE 6. EFFECT OF CORTISONE AND METHYLANDROSTENEDIOL (M.A.D.) UPON GRANULOMA FORMATION INDUCED BY SUBCUTANEOUS COTTON PELLETS IN RATS

Treatment	Per cent increment in dry weight cotton pellet
A. Systemic steroids	
Untreated controls	146 ± 6.6*
M.A.D. 3 mg. daily for 6 da.	123 ± 9.8
Cortisone acetate 2 mg. daily for 6 da.	70 ± 5.6
Cortisone acetate 2 mg. + M.A.D. 3 mg. daily for 6 da.	70 ± 3.5
B. Local Steroids	
Cortisone acetate 0.25 mg.	123†
Controls	162
M.A.D. 0.75 mg.	143
Controls	143
Cortisone acetate 0.25 mg. + M.A.D. 0.75 mg.	98‡
Controls	139

* Standard error.
† Difference between controls and treated animals, $P = 0.05$.
‡ Difference between controls and treated animals, $P < 0.01$.

It is apparent that M.A.D. alone had no effect upon the production of edema, and that cortisone was not less effective in presence of the androgen.

In another experiment, cotton pellets were implanted subcutaneously and the amount of granuloma tissue formed at the site of the pellet was determined by removing the pellet together with the granuloma around it after 6 days, according to the technique described by Meier, Schuler and Desaulles (1950). This reaction is inhibited by cortisone, either administered systemically or by local application (incorporation of the steroid in the pellet). In the test of the local action of the steroid, a control pellet is placed on one side of the animal, and a pellet containing steroid on the opposite side. Thus, each animal serves as its own control. Table 6 gives the results by both methods of administration. It is evident that M.A.D. does not have any influence upon the amount of granuloma formed, nor does it antagonize the inhibition of the reaction by cortisone.

Both these experiments make it clear that although M.A.D. antagonizes the effect of cortisone in producing adrenal atrophy, it does not antagonize the anti-inflammatory action of cortisone.

DISCUSSION

Although cortisone has proven to be useful in a variety of conditions, it exerts certain hormonal effects which do not contribute to its therapeutic value, and which, indeed, it would be desirable to avoid, if possible. Among these is adrenal atrophy. This investigation was undertaken with the thought that if one or more of these unwanted effects could be counteracted without diminishing the desired actions of the hormone, the usefulness of cortisone might be enhanced. This hope seems to have been at least partially realized in these animal experiments, although the therapeutic applicability of these results has not as yet been demonstrated. The hyperadrenocortinism produced in patients by large doses of cortisone resembles in many respects the findings in Cushing's syndrome. Albright (1942–43) found androgens to be of value in correcting many of the manifestations of this disease. Pearson (1951) also reported that testosterone would counteract some of the effects of cortisone on normal tissue.

Both testosterone and methylandrostenediol exerted a sparing action upon the adrenal cortices of animals treated with cortisone. Both these compounds are androgens, and both also have anabolic action as shown by growth-promotion and nitrogen balance tests (Kochakian, 1949; Homburger *et al.*, 1950). Methylandrostenediol, however, has been reported to be relatively high in the anabolic action typical of androgens, but low in androgenic potency (Gordan *et al.*, 1951). It is possible that the sparing action of these steroids on the adrenal cortex of cortisone-treated rats is a consequence of their anabolic action. In the present experiments, no such action could be demonstrated upon the growth-suppressing action of corti-

sone. However, it is not necessary to assume that steroids have a qualitatively similar action upon metabolic phenomena in all the tissues of the body; indeed, there is good evidence that cortisone has opposite metabolic effects upon different tissues (Kochakian and Robertson, 1951). It is therefore not unreasonable that our experiments might show an anabolic action of M.A.D. on the adrenals even though they did not demonstrate such action on the body as a whole.

In the intact animals, it might be argued that M.A.D. could act as a stress to effect the release of ACTH, and adrenal atrophy could be prevented by release of this hormone from the hypophysis. Alternatively, M.A.D. might block the inhibiting effect of cortisone upon the release of ACTH. The results in hypophysectomized rats deny both these alternatives. The repair of adrenals after hypophysectomy when both cortisone and M.A.D. are used, but not when either is administered alone leads to the surprising conclusion that both cortisone and the androgen are necessary for this result. Also, in the experiments on adrenal repair after establishment of adrenal atrophy by cortisone, M.A.D. brings about some repair if it is administered while cortisone is being given. On the other hand, when M.A.D. is given alone; i.e., after discontinuance of cortisone, adrenal repair does not proceed more rapidly than if no steroid is being given. Therefore, in this experiment as in the experiment with hypophysectomized rats, it appears that it is the combination of the two steroids which is effective against adrenal atrophy, rather than either one alone. On the other hand, no combination of the two steroids was used which gave adrenals larger than those of nomal intact rats (several dosage ratios were tried which were not included in the tables) nor did M.A.D. alone in intact rats produce large adrenals.

SUMMARY

1. Testosterone propionate or methylandrostenediol administered in conjunction with cortisone acetate in rats prevented the adrenal atrophy which occurred when cortisone alone was given. Adrenal cortices of rats given both the androgen and cortisone were essentially normal in size and histological appearance.

2. Combination therapy with methylandrostenedial and cortisone produced considerable repair of the adrenals of hypophysectomized rats. Neither steroid alone produced repair of adrenals in such animals.

3. Adrenals made atrophic by cortisone injection could be partially repaired by administration of methylandrostenediol while cortisone treatment was continued. However, adrenal recovery after discontinuance of cortisone was not hastened by injection of the androgen.

4. Methylandrostenediol administered with cortisone did not inhibit the anti-inflammatory action of cortisone.

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GONADOTROPINS OF THE PITUITARY GLAND AND THE URINE OF THE ADULT HUMAN FEMALE

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IN A previous paper (Bahn *et al.*, 1952), a study of the gonadotropins of the pituitary gland and the urine of the adult human male was made. The purpose of this paper is to present similar data on the pituitary gland and the urine of the adult human female.

I. MATERIALS AND METHODS

A. Animals. Male and female Wistar rats (30 to 40 days old) were hypophysectomized; injected, some with pituitary homogenate and others with urine concentrate; and subsequently studied as described previously.

B. Pituitary tissue. The pituitary gland of 2 adult human females was obtained within three to four hours following death. Neither of the patients presented any obvious endocrinopathy. Menstruation had occurred at regular monthly intervals. The ages of the patients were 38 and 46 years. There was no prolonged terminal illness. The combined wet weight of the anterior lobes of the pituitary glands was 1,219 mg.

C. Urine. A number of complete twenty-four hour specimens of urine were obtained from 5 healthy menstruating adult females during all phases of the menstrual cycle.

II. RESULTS

A. Pituitary homogenate in female animals. The results observed in hypophysectomized female rats receiving anterior pituitary homogenate are listed in Table 1. The data indicate that both follicle-stimulating (FSH) and luteinizing (LH, ICSH) hormones are present in the normal adult human female hypophysis. Neither early follicular growth nor repair of the ovarian interstitial "deficiency cells" was observed until a total dose of 3 mg. of the pituitary homogenate was given.

Uterine and ovarian enlargement occurred at a dose of 10 mg. The increase in ovarian weight was associated with the presence of many large cystic follicles. There was only slight luteinization of the granulosa cells while the interstitial and thecal cells had undergone marked lutein cell transformation. The response to 30 mg. of anterior pituitary homogenate

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TABLE 1. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals			Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	4	0	5.6	0.10	15.0	0.28	0	0	D	0
Intact	6	0	18.6	0.18	118.3	1.17	+++	+	R	+
Hyp.	4	1	8.0	0.12	21.8	0.32	0	0	D	0
Hyp.	3	3	9.2	0.14	22.9	0.35	+	0	R	0
Hyp.	4	10	38.8	0.54	98.8	1.37	++++	0	L	0
Hyp.	1	30	81.2	1.35	78.4	1.31	++++	+	L	+

Abbreviations: Hyp. = Hypophysectomized; mg./gm. = Organ weight per gram body weight; D = Deficient; R = Repaired; I.T. = Interstitial tissue; 0 = absent; + = Present; L = Luteinized; V.O. = Vaginal opening; F.D. = Follicular development.

was characterized by additional ovarian enlargement and the formation of numerous corpora lutea.

In terms of the previously defined units, there is approximately 1 unit of FSH and 1 unit of LH per 3 mg. of pituitary homogenate, or a total of about 200 units of both hormones per pituitary of the adult human female.

B. Pituitary homogenate in male animals. Table 2 lists the results on hypophysectomized male rats. At the 3 mg. dose level FSH activity was indicated by a doubling of testicular weight, an increase in tubular

TABLE 2. EFFECT OF HUMAN PITUITARY HOMOGENATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals			Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Dose (mg.)	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	2	0	13.9	0.22	15.0	0.23	47.3	0.74	0.10	+	0	0	U.
Intact	5	0	62.2	0.58	41.0	0.38	132.2	1.22	0.22	++++	+	+	Di.
Hyp.	3	1	27.3	0.39	22.5	0.32	75.6	1.09	0.14	++	0	0	U.
Hyp.	3	3	45.8	0.64	41.1	0.57	115.4	1.61	0.16	+++	0	0	Di.
Hyp.	2	10	54.2	0.77	46.5	0.66	140.1	2.00	0.15	+++	0	0	Di.
Hyp.	3	30	52.7	0.68	60.1	0.78	163.9	2.13	0.16	++++	+	0	Di.

Abbreviations: M.T.D. = Mean tubular diameter (millimeters); S.A. = Spermatocytic proliferation; St. = Spermatid; Sz. = Spermatozoa; I.T. = Interstitial tissue; U. = Undifferentiated; Di. = Differentiated; Others = See table 1.

diameter and active spermatocytic proliferation as compared with the controls. Thirty milligrams of pituitary homogenate tripled the testicular weight, but did not completely restore the normal testicular histologic pattern. Only the testis of the intact control animals exhibited spermatozoa.

At the 3 mg. dose level LH activity was indicated by the appearance of well-differentiated Leydig cells. Nevertheless, indirect evidence of Leydig cell function was present at the 1 mg. dose level as judged by the weights and results of histologic examination of the prostates and seminal vesicles. The amount of homogenate necessary to repair the deficient Leydig

cells as judged by histologic evidence was 3 mg. The same amount also brought about a distinct response in the tubular apparatus of the testis as judged by testicular weight, tubular diameter and spermatogenic activity. Again, roughly equal FSH and LH activities, about 200 units of each, are present in the adult female pituitary.

C. Urine in female animals. Table 3 shows the effect of a concentrate equivalent to 1,200 cc. of pooled urine from adult females. The presence of

TABLE 3. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED FEMALE RAT

Animals				Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	F.D.	Corpora lutea	I.T.	
Hyp.	2	0	0	7.3	0.11	16.3	0.23	0	0	D	0
Hyp.	3	+	0	8.7	0.12	16.3	0.23	0	0	D	0
Hyp.	5	+	+	20.2	0.30	76.8	1.15	++++	0	D	+
Intact	5	0	0	18.4	0.16	83.9	0.73	+++	+	R	+

Abbreviations: Same as in table 1.

FSH was shown by the increased ovarian weight and the marked response of the ovarian follicles. The latter were large cystic structures without signs of luteinization. Ova were present within these follicles. Both ovarian and uterine enlargements were about equal to that brought about by 3 to 10 mg. of pituitary homogenate.

The presence of LH was shown by the copious secretion of estrogen, resulting in uterine enlargement. However, since there was no repair of the interstitial tissue of the ovary, less than 1 unit of LH as previously defined was present in 1,200 cc. of urine.

D. Urine in male animals. Table 4 lists the weights of organs of hypophysectomized male rats receiving urinary concentrate. Little change oc-

TABLE 4. EFFECT OF URINARY CONCENTRATE ON THE HYPOPHYSECTOMIZED MALE RAT

Animals				Prostate		Seminal vesicles		Epididymis		Testicular histology				
Condition	No.	Cortisone	Concentrate	mg.	mg./gm.	mg.	mg./gm.	mg.	mg./gm.	M.T.D.	S.A.	St.	Sz.	I.T.
Hyp.	4	0	0	6.5	0.09	8.6	0.12	49.5	0.69	0.13	+	0	0	U.
Hyp.	5	+	0	6.6	0.10	8.9	0.14	41.9	0.66	0.13	+	0	0	U.
Hyp.	5	+	+	9.4	0.14	10.9	0.16	76.0	1.09	0.16	+++	+	0	U.
Intact	5	0	0	65.7	0.55	44.3	0.37	171.3	1.44	0.23	++++	+	+	Di.

Abbreviations: Same as in Table 2.

curred in the number or the size of the interstitial cells as compared to the hypophysectomized control animals. Nevertheless, the weight of the ventral prostate, epididymis and seminal vesicles was distinctly increased over that of the hypophysectomized controls. Moreover, microscopic examination of the ventral prostate and seminal vesicles gave evidence of

androgenic stimulation. The androgenic effect without repair of the Leydig cells indicated that less than 1 unit of LH was present in 1,200 cc. of urine.

The testicular weight was increased to about twice that of the hypophysectomized control. Spermatogenesis had progressed to the spermatid stage. The increase in testicular weight and in mean tubular diameter was generally equivalent to that produced by more than 3 but less than 10 mg. of pituitary homogenate.

III. COMMENT

Our previous study showed that while the anterior pituitary gland of the male contained about 1 unit of both FSH and LH activity per 3 mg. of wet tissue, the male urine contained more FSH activity than LH activity.

The pituitary gonadotropins of the adult human female are strikingly similar to those of the adult male. The concentration of FSH and LH of the anterior lobe of the hypophysis of the adult human female is the same as that of the male. Since the pituitary glands of the females are heavier than those of the males, the total amount of the gonadotropins per gland was greater in the females than in the males. This is in accord with the reports of Rasmussen (1938) and of Henderson and Rowlands (1938).

The urinary gonadotropins of the adult human female are similar to those of the adult male. The accessory reproductive organs of the hypophysectomized male rats were distinctly enlarged after injection of female urine. This increase in weight was not present in similar experiments following the injection of adult male urine. These data suggest that adult female urine contained more LH than adult male urine. That there is a greater LH content in urine of the female than of the male has been suggested by Lloyd *et al.* (1949) on the basis of ovarian hyperemia tests. Moreover, McArthur (1952) reported greater increases in the weight of the prostate and seminal vesicles of hypophysectomized male rats injected with an extract of female urine than were observed in our animals. Differences between her results and those recorded in this paper may have been brought about by some inhibition of prostatic growth by the cortisone which was administered to our rats during the course of the experiment.

Pedersen-Bjergaard and Tønnesen (1948a, b) studied the urinary gonadotropins in a large series of adult men and women. In terms of uterine enlargement of the intact immature female rat, their average urinary gonadotropin values were 6 units per day for the adult male and 9 units per day for the adult female. This small difference might be accounted for on the basis of the augmentation of FSH (Fevold, 1941) by the slightly increased LH found in the urine of females.

It is apparent that in the adult human, both male and female, there is a marked qualitative difference between the gonadotropins of the hypophysis and those extracted from the urine. A number of possible explanations have been proposed but further study is necessary to demonstrate which of these or other possibilities is responsible for the disparity.

IV. SUMMARY

The pituitary gland of the adult human female contains equal activity of FSH and LH. Urine from the adult female, however, contains more FSH activity than LH activity.

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IN VITRO BIOASSAY OF CORTICOTROPHIN

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WE HAVE observed that pituitary adrenocorticotrophic hormone (corticotrophin), added *in vitro*, stimulates the formation of corticoids by isolated rat adrenals and that the stimulation is approximately proportional to the amount of corticotrophin added (Saffran, Grad and Bayliss, 1952). This observation has been adapted to the bioassay of corticotrophin.

METHOD

Four adult Sprague-Dawley rats, of either sex, are used for each assay. The adrenals are prepared as previously described (Saffran, Grad and Bayliss, 1952). Care must be taken to keep the pair of adrenals from each rat together during the preparation. Four adrenals, one from each pair, are weighed together, bisected, and placed into a Warburg vessel containing 2 ml. of the Krebs-Ringer-phosphate-glucose medium. The remaining four adrenals are then weighed, bisected, and placed into a second Warburg vessel. The flasks are attached to Warburg manometers, flushed with oxygen, and are shaken at 38° for 1 hour. At the end of this period (the preincubation period) the media from both flasks are removed with suction and discarded. Both flasks are then recharged with fresh medium, and a small volume (about 0.1 ml.) of the standard corticotrophin solution is added to one flask and the unknown corticotrophin solution to the other. Approximately the same activity of corticotrophin should be added to both flasks. The flasks are again flushed with oxygen and shaken at 38°, this time for 2 hours. At the end of this period the media are analyzed separately for corticoids by a slight modification of the method previously described (Saffran, Grad and Bayliss, 1952). The modification consists of passing the chloroform extract through filter paper instead of drying with sodium sulfate. The filtered chloroform extract is then taken to dryness as previously described.

The *amount* of corticotrophin is expressed as units of the standard preparation and mg. of the unknown per 100 mg. of adrenal tissue in the flask. The *activity* of the corticotrophin is expressed as μg. of corticoids produced by 100 mg. of adrenals in 2 hours.

The potency of the unknown sample is calculated as follows:

$$\text{Potency (units per mg.)} = \frac{\text{units of standard corticotrophin x activity of unknown}}{\text{mg. of unknown corticotrophin x activity of standard}}$$

The procedure is outlined in the sample protocol. In actual practice several levels of standard and unknown preparations are matched for each determination of potency.

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SAMPLE PROTOCOL

COMPARING ARMOUR'S CORTICOTROPHIN WITH THE U.S.P. REFERENCE STANDARD

	Standard	Unknown
Weight of 4 adrenals, mg.	48	45
Tissue added to flask	9:58 A.M.	10:00 A.M.
Preincubation period	10:10 to 11:10 A.M.	
Corticotrophin added	U.S.P.	Armour
ml.	0.1	0.1
units or mg./flask	0.025 unit	0.012 mg.
units or mg./100 mg.	0.053 unit	0.027 mg.
Second incubation period	11:30 A.M. to 1:30 P.M.	
Observed optical densities:		
225 mμ	0.285	0.285
240 mμ	0.260	0.254
255 mμ	0.174	0.167
Calculated O.D.	$.260-\frac{.285+.174}{2}=0.031$	$.254-\frac{.285+.167}{2}=0.028$
Total μg. corticoids	5.6	5.1
μg. corticoids/100 mg.	11.75	11.3

Potency of corticotrophin, Armour $=\frac{0.053\times11.3}{0.027\times11.75}=1.93$ units/mg.

EXPERIMENTAL

Extraction of corticoids

A methanolic solution of the residue from a chloroform extract of the medium alone absorbs light in the ultraviolet at the wave lengths used to measure the corticoids (Fig. 1). Although the absolute value of the blank absorption varies somewhat, the shape of the curve is almost invariably the same. Because the blank curve approaches a straight line it is possible to use the calculation of Allen (1950) to estimate the absorption at 240 mμ caused by the α,β-unsaturated ketonic grouping of the corticoids. The absorption curve of a chloroform extract of the incubation medium of rat adrenals kept at 2° for 2 hours in the presence of corticotrophin is very similar to the blank. Figure 1 includes the curve of an extract of the incubation medium of adrenals kept at 38° with corticotrophin present and the absorption curve of cortisone for comparison with the blank curves.

Attempts to eliminate the high blank absorption by replacing the chloroform with methylene chloride, ethyl acetate, heptane, or ether failed because these solvents either yielded higher blank values than chloroform or because they did not extract the corticoids from an aqueous medium.

Preincubation

In this paper "preincubation" means the incubation of the adrenals for

1 hour in medium alone before the incubation in fresh medium containing corticotrophin. Preincubation increases the sensitivity of isolated rat adrenals to added corticotrophin about 20–25 times. This is illustrated in

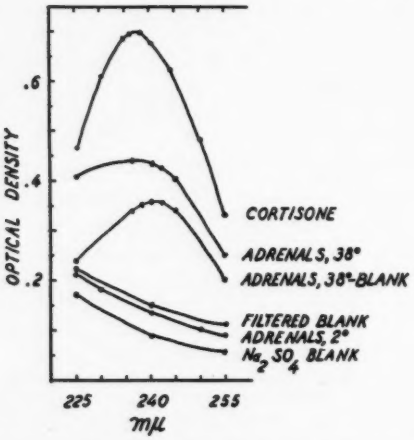

FIG. 1. Absorption curves of methanolic solutions of the residues of chloroform extracts of the incubation medium. "Na_2SO_4 BLANK" was obtained by drying the chloroform extract of 2 ml. of Krebs-Ringer-phosphate-glucose medium with anhydrous sodium sulfate. "FILTERED BLANK" was obtained by passing the chloroform extract through filter paper instead of drying it with sodium sulfate. "ADRENALS, 2°" was obtained by extracting with chloroform the incubation medium of adrenals kept with corticotrophin for 2 hours at 2°. "ADRENALS, 38°" was obtained with a chloroform extract of the incubation medium of adrenals kept with corticotrophin for 2 hours at 38°. When the corresponding blank extraction curve is subtracted from it, the curve "ADRENALS, 38°—BLANK" results. This curve may be compared with that of a methanolic solution of cortisone.

Fig. 2 which compares the formation of corticoids by rat adrenals in the presence of varying amounts of corticotrophin, with and without the preincubation period. Table 1 compares the production of corticoids by adrenals from the same group of animals in the presence of a small amount

TABLE 1. EFFECT OF PREINCUBATION ON THE FORMATION OF CORTICOIDS

	Corticotrophin added	Corticoids formed
	units/100 mg.	μg/100 mg./2 hrs.
Preincubated	0.11	32.6
Not preincubated	0.11	0
Preincubated	0.13	24.7
Not preincubated	0.13	<7.3

of corticotrophin, with and without preincubation. Only the preincubated glands produce significant amounts of corticoids.

Figure 2 shows that the response of preincubated rat adrenals to increasing amounts of corticotrophin reaches a maximum with the production of 40–50 μg. of corticoids per 100 mg. of adrenals per 2 hours. In the bioassay it is therefore necessary to add the amount of corticotrophin that results in

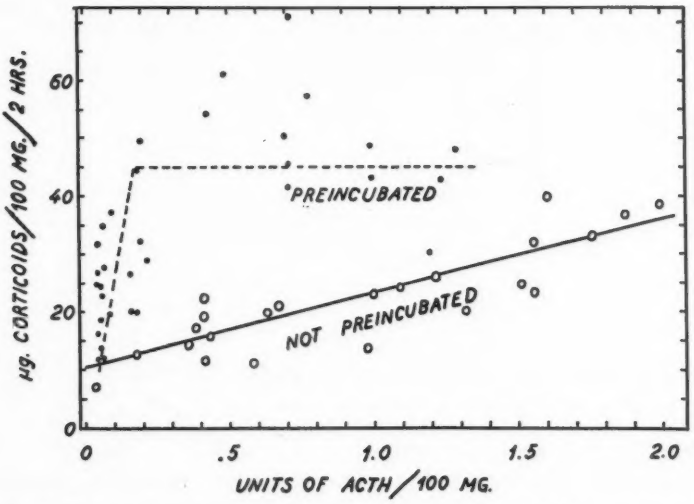

FIG. 2. Effect of preincubation on the production of corticoids by bisected rat adrenals in the presence of increasing amounts of corticotrophin. The curve labelled "PREINCUBATED" was drawn by inspection. That labelled "NOT PREINCUBATED" was calculated by the method of least squares.

the formation of 10–30 μg. of corticoids. The measurement of less than 10 μg. of corticoids is uncertain.

Preincubation also decreases the production of corticoids in the absence of added corticotrophin (Table 2). This is important in reducing the error of the bioassay, in which the control value is not measured.

TABLE 2. CORTICOID FORMATION WITHOUT ADDED CORTICOTROPHIN

1st hour	Next 2 hours
μg. corticoids/100 mg./hour	
4.0	0.1
4.7	1.6
2.5	3.0
16.0	2.0

Rate of corticoid formation

The rate of formation of corticoids, in the presence or absence of added corticotrophin, is approximately constant for 4 or 5 hours (Fig. 3).

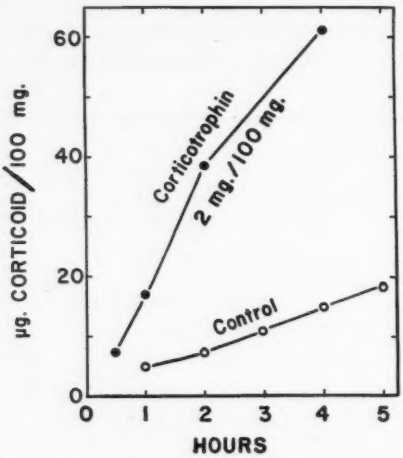

FIG. 3. Rate of formation of corticoids by bisected rat adrenals in the presence and absence of added corticotrophin.

Persistence of corticotrophin effect

Rat adrenals, incubated for 1 hour with corticotrophin, produce increased amounts of corticoids even after two thorough rinsings with the medium (Fig. 4).

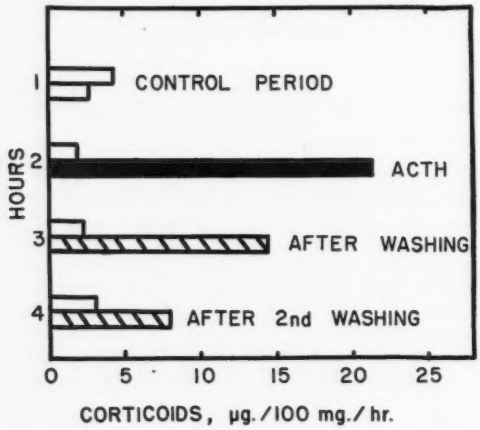

FIG. 4. Persistence of the effect of added corticotrophin after washing of the adrenal tissue.

Incubation in blood

Because of the reported greater production of corticoids by beef adrenals when perfused with whole blood than with plasma (Hechter *et. al.*, 1951), bisected rat adrenals were incubated in citrated homologous blood instead of the usual medium. In this experiment a mixture of 5% carbon dioxide in oxygen was used as the gas phase. At the end of 2 hours little or no corticoids were found in a chloroform extract of the blood (Table 3).

TABLE 3. EFFECT OF CHANGES IN CONDITIONS OF INCUBATION ON THE FORMATION OF CORTICOIDS

Conditions	Corticotrophin added	Corticoids formed	Oxygen consumption
	units/100 mg.	μg./100 mg. per 2 hrs.	μM/100 mg. per 2 hrs.
Citrated blood and 5% CO_2	0	4.1	
	1.7	3.4	
	0	0	
	1.7	9.4	
	0	0	
	1.6	0	
Oxygen	1.7	32.1	12.5
Air	1.7	23.9	3.9
Nitrogen	1.6	0	0
	1.7	0	0
Bisected	1.5	24.8	11.6
	1.7	30.0	12.2
Minced	1.5	28.9	7.6
	1.6	21.6	7.2
Homogenized	0	0	1.0
	1.0	0	1.2

N.B. These samples were *not* preincubated.

Oxygen requirement

The oxygen requirement of the system that forms corticoids was investigated by incubating rat adrenals in air and in nitrogen. The formation of corticoids was somewhat lower in air than in oxygen, but no corticoids were formed during the anaerobic incubation in nitrogen (Table 3).

Mincing and homogenization

Mincing the adrenal tissue with scissors does not affect the formation of corticoids, but there is no formation in an unfortified homogenate of adrenals (Table 3).

Agreement between duplicates

Four adrenals, one from each of four rats, form about the same amount of corticoids as the four remaining adrenals from these animals, when the two lots are incubated under the same conditions (Table 4).

TABLE 4. AGREEMENT BETWEEN DUPLICATE INCUBATIONS

	Corticotrophin added	Corticoids formed	
	units/100 mg.	μg./100 mg./2 hrs.	
Not preincubated	0	4.2	2.6
	0	1.6	1.9
	0	3.3	4.2
	1.0	12.6	9.9
	2.5	18.5	16.0
	2.6	20.3	16.1
Preincubated	0	5.8	4.6
	0	3.8	7.4
	0	3.0	1.5
	0.8	32.3	35.8

RESULTS

The *in vitro* bioassay method was used to determine the potency of several preparations of corticotrophin. The values agree very well with the estimated potencies based on the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury (1948) (Table 5).

TABLE 5. POTENCY OF CORTICOTROPHIN PREPARATIONS BY THE *in vitro* BIOASSAY

Preparation	Estimated potency by *in vitro* assay	Claimed potency*
	U.S.P. units/mg.	U.S.P. units/mg.
Armour, lot J16609	1.6	2
	1.9	
	1.9	
Astwood, crude	1.6	2
	2.0	
	1.8	
Astwood, purified	50.1	60–100
	52.6	(average, 80)
	56.2	
	66.6	
Canada Packers, lot 2-C	3.1	3–4
	2.7	
	3.5	
Canada Packers, lot FU	3.0	3–4
Canada Packers, lot I	2.9	4–5
(deteriorating sample because of high moisture content)	2.7	

* Estimated by the method of Sayers, Sayers and Woodbury (1948). Values supplied by the donors of the corticotrophin preparations.

DISCUSSION

Although the optimum conditions for the *in vitro* assay method have not been established yet, it yields results that agree very well with the adrenal ascorbic acid depletion method. The *in vitro* method has several advantages. Firstly, the same animals are used to provide tissue for both the standard and unknown preparations of corticotrophin. Secondly, there is

no need to maintain a supply of hypophysectomized animals because in the *in vitro* assay the adrenals are effectively removed from the influence of the pituitary. Thirdly, a known function of corticotrophin, namely, the control of the secretion of adreno-cortical hormones, is used as the basis of the assay. A disadvantage of the method is its relative insensitivity, but this is of little consequence when the potency of a preparation of corticotrophin is being determined.

The method is based on an assumption for which there is no direct evidence. It is, that a given group of adrenals, incubated together, will produce an amount of corticoids that is proportional to the amount of corticotrophin added. In the case of the adrenals (Fig. 2) that were not preincubated, the response to increasing amounts of corticotrophin seems to be more or less linear. After preincubation, the adrenal response curve is very steep, but for the assay it is assumed to be linear as well. That this assumption is valid to some extent is shown by the good agreement among replicate assays of the various preparations of corticotrophin (Table 5).

Both adrenals of a rat respond similarly to corticotrophin (Table 4). This allows the comparison of standard and unknown preparations of corticotrophin on similarly-reacting tissue. If homogeneous preparations of adrenal tissue can be shown to respond to corticotrophin like bisected adrenals, it would be possible to divide the tissue into more than two aliquots and allow the comparison of more than two samples using the same tissue. That this might be so is indicated by the response of an adrenal mince to corticotrophin (Table 3).

Preincubation increases the sensitivity of the adrenals to corticotrophin about 20–25 times. During the preincubation period a large proportion of the ascorbic acid is leached from the adrenal tissue (Saffran, Bayliss and Webb, 1951) and the tissue may have had the time to recover from any previous stimulation by corticotrophin in the animal. However, there is, as yet, no evidence for any explanation of the effect of preincubation.

The effect of corticotrophin on the adrenal *in vitro* persists after washing the tissue (Fig. 4). This observation is similar to that of Stadie and his group (Stadie, Haugaard and Marsh, 1951) on the *in vitro* effect of insulin. It is not possible now to state whether the corticotrophin is "bound" by the adrenal tissue.

SUMMARY

The stimulation by corticotrophin of the production of corticoids by bisected rat adrenals *in vitro* has been adapted to the assay of corticotrophin. The sensitivity of the method is greatly increased by the preincubation of the adrenal tissue without added corticotrophin for 1 hour, prior to exposure to corticotrophin. The range of the assay is approximately 0.04 to 0.1 U.S.P. units of corticotrophin per 100 mg. of adrenal tissue.

There is very good agreement between the *in vitro* bioassay and the adrenal ascorbic acid depletion method.

The stimulation by corticotrophin of the production of corticoids by rat adrenals persists after washing the tissue to remove the corticotrophin.

Corticotrophin stimulates the production of corticoids in an atmosphere of air as well as in oxygen, but the rat adrenal does not form corticoids in nitrogen.

An adrenal mince responds to added corticotrophin like bisected adrenals, but unfortified homogenates do not produce corticoids.

Acknowledgments

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NEUROHUMORAL CONTROL OF THE PITUITARY IN THE FOWL

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IN THE domestic fowl approximately 23 to 28 hours elapse between ovulation and the laying of the finished egg. During the intervening period the ovum acquires albumen in the magnum and shell membranes in the isthmal portion of the oviduct. This is accomplished in 4.5–5.0 hours and the remaining time is spent in the shell gland where the calcifierous shell is deposited. Warren and Scott (1935) showed that ovulation does not usually occur while there is an egg in any portion of the oviduct. These facts and the finding that, in hens laying daily, ovulation takes place within 30–60 minutes after oviposition, led to the assumption that an intricate timing mechanism may be involved to synchronize reproductive events in chickens. An observation in this laboratory that injury to the oviduct led to a profound upset in the laying cycle of hens caused more intensive analysis of the relationship between events in the oviduct and the endocrine function of the pituitary.

Rothchild and Fraps (1949) have shown that the ovulating hormone (LH) is released about 4 to 8 hours prior to ovulation. Thus, factors which may be involved either in stimulating or in inhibiting release of LH must exert their effects several hours prior to ovulation. Since there is only a relatively short interval between oviposition and the succeeding ovulation it becomes apparent that the events leading to ovulation usually occur while the egg is in the shell gland.

These observations led to the experiments reported here.[1] Initially, such irritants as bits of key chain, cork or pellets of paraffin were placed in the lumen of the magnum. These foreign bodies were attached to coarse surgical thread which in turn was passed through the wall of the oviduct and tied securely so as to prevent evacuation of the irritants from their original location in the lumen due to peristaltic movements of the oviduct. When paraffin pellets were used, they were soon softened by body

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[1] We wish to acknowledge the help of Dr. H. W. Norton in the design of some of the experiments and their statistical analysis. We thank Dr. H. W. McShan and R. K. Meyer (Univ. of Wisconsin) for the ISAP, Mr. Irby Bunding of Armour and Co. of Chicago for the LH and Dr. R. E. Waterman of the Schering Corp., Bloomfield, N. J. for the progesterone.

heat, torn loose from the thread fastening them to the oviduct wall and passed out. It was thus noticed that the thread alone, which remained after the paraffin pellet was evacuated, provided sufficient stimulus to cause profound changes in the laying cycle of these hens.

ANIMALS AND TECHNIQUES

The birds used were sexually mature hens less than two years old, of the White Leghorn and New Hampshire breeds. They were trapnested prior to the experiments to determine their normal rate of production. Only regularly laying birds were used. They were maintained in individual cages in laying batteries and allowed free access to a standard laying mash and water.

At the time of operation the birds were anesthetized with nembutal, and the oviduct was exposed through an incision in the abdominal wall. A loop of coarse surgical thread was passed through the wall of the oviduct into the lumen of the magnum in such a manner as to extend longitudinally through approximately an inch of the lumen. The thread was again passed through the wall of the magnum and the two ends of the thread were tied externally. In placing the loop care was taken to assure that the lumen of the oviduct was not obstructed.

In a second group of birds the oviduct was exposed in the same manner but no loop of thread was placed in the oviduct (sham operated controls). Birds in both groups were weighed at the start and at the end of the experiment; their combs were measured on alternate days and individual daily records of egg production were kept.

The hormones used in some of the experiments included progesterone in oil; a partially purified gonadotrophic preparation containing both follicle stimulating (FSH) and luteinizing hormones (LH) called ISAP; and a highly purified preparation of LH. The gonadotrophic hormones were dissolved in water and injected intravenously. One single injection of ISAP was made while the LH was injected twice daily until ovulation occurred. The progesterone was injected subcutaneously. Control animals were injected with equal amounts of oil or water.

The laid egg was the criterion used for determination of ovulation, with the exception of one experiment involving ISAP in which the hens were sacrificed and the number of ovulations verified at autopsy. At the conclusion of some of the experiments the birds were sacrificed, the ovaries and oviducts removed, weighed, measured and described with regard to their physiological condition.

After the basic physiologic effects on the ovary and the oviduct of the operation described were established, the hens were no longer sacrificed since the laid egg was taken as the evidence for success of the treatment when hormones were injected.

RESULTS

In order to establish the quantitative and qualitative effects of the loop on the laying cycle of the birds, on their reproductive organs and their comb size, groups of birds were killed 5, 10, 15, 20, 25 and 30 days after the operation. Eight of the birds destined to be killed at the different intervals in each group had a loop in the magnum, and eight served as sham-operated controls. Due to mortality at operation and during the course of the experiment some of the groups did not contain the full complement of eight birds at the time of autopsy (Table 1).

The most striking effect of the loop in the magnum was that it almost completely inhibited ovulation during the first 25 days after the operation. There were a few sporadic ovulations which were widely spaced (Table 1). Eight of the 33 birds with a thread in the magnum laid a total of 12 eggs (1.5 eggs per bird). That the operation itself, in absence of the loop of thread in the magnum, is not responsible for the interruption of laying is seen from the fact that during the same period 29 sham-operated hens laid 121 eggs (5.5 eggs per bird).

TABLE 1. EFFECT OF A THREAD IN THE MAGNUM ON THE SIZE OF OVARIES, OVIDUCTS AND COMBS, AND THE RATE OF OVULATION. OBSERVATIONS MADE AT 5-DAY INTERVALS

Treatment	Days from operation to autopsy	Number of birds	Number of birds laying	Total number eggs laid	Ovary weight (gms.)	Oviduct weight (gms.)	Comb area (cm.)	Average size of follicles (cm.)	Average number of follicles of ovulatory size (30 mm.)
L	5	6	1	2	45	53	25	19	0.75
			p = 0.15*	SE	8.7	6.2	5.1	1.4	
S	5	5	3	7	48	55	33	22	0.40
					8.0	2.0	5.6	1.0	
L	10	8	2	4	48	53	23	21	0.40
			p = 0.05*		5.9	4.1	1.6	0.8	
S	10	7	5	12	51	63	23	23	0.6
					3.5	4.3	4.0	1.1	
L	15	5	1	1	56	65	27	22	1.0
			p = 0.001*		5.3	3.5	1.4	1.3	
S	15	7	7	44	58	67	32	23	1.0
					3.4	3.3	3.8	1.1	
L	20	7	2	2	68	68	31	24	1.71
			p = 0.05*		4.8	3.7	4.4	1.0	
S	20	5	3	24	52	47	24	22	1.25
					10.9	5.9	2.0	1.4	
L	25	7	2	3	17	42	29	all	0.00
			p = 0.01*		2.5	3.9	2.3	atretic	
S	25	5	4	34	43	30	25	22	1.12
					10.4	13.5	3.6	1.5	
L	30	6	3	31	59	69	30	—	—
			p = 0.06*		6.8	8.6	1.4		
S	30	6	5	55	52	60	34	—	—
					6.4	4.4	3.8		

L—hens, with thread in magnum of oviduct.
S—sham operated controls.
*—levels of significance of differences in egg production between S and L.
SE—standard error of means.

In all subsequent experiments essentially the same results were found as those discussed here. The presence of an irritant in the magnum modified the laying cycle of all hens treated, although not all the animals treated responded by complete cessation of laying. In most hens, laying was suppressed completely (in 75% of the hens in one experiment, in 58% in another). Most remaining hens laid occasional eggs, but few continued to lay at an almost normal rate. In most cases the rate of production was re-

duced very drastically as compared to the almost unimpaired rate of production of the sham-operated group.

The second important observation regarding the presence of the thread in the oviduct was that 30 to 40 days after the operation laying was gradually resumed and eventually became normal (Table 1, 30-day group).

It is also interesting that the loop affected neither the size, the morphological condition of the ovary, nor that of the oviduct or the comb (Table 1). Ova which were present in the ovary at the time of the operation seem to be maintained without degenerating during the first 20 days after the operation, thus accounting for the non-reduction in ovarian weight (Table 1). This is true not only for number of follicles but also for size. In this particular experiment atresia of follicles set in at about 25 days and was observed in all seven hens in this group. At 30 days, when the next group was killed, the ovaries were found to be normal and three of the six hens in this group were laying. Statistical analysis showed that the differences in production between the sham-operated and the hens with a thread in the magnum were significant in all groups except the 5-day and the 30-day groups (Table 1).

These observations led to the assumption that the irritant in the oviduct did not depress pituitary function because the size of the ovary (number and size of follicles) was not reduced for as long as 20 days after the operation. Since neither the ovary nor the oviduct or the comb were reduced in size, it appeared that the ovary continued to secrete androgen and estrogen at normal rates.

These facts led to the theory that the presence of an irritant in the uterus specifically prevented the release of sufficient amounts of LH from the pituitary to cause ovulation without, however, interfering with the other endocrine functions of the pituitary. The mechanism of this inhibiting action on the secretion of LH was thought to be neuro humoral in nature and experiments were conducted to test this assumption.

In order to establish the relationship of the presence of the thread to the inhibition of ovulation, two experiments were designed. In the first, 12 hens which had ceased laying after the placing of the thread were injected intravenously with a crude gonadotrophic preparation, ISAP, containing

TABLE 2. NUMBER OF OVULATIONS OCCURRING WITHIN 72 HOURS AFTER ONE INTRAVENOUS INJECTION OF 0.25 GRAM EQUIVALENT OF ISAP (ISAP 16: 43.6 MGS. EQUIVALENT TO 1 GRAM OF ACETONE-DRIED SHEEP PITUITARY POWDER)

Results	Number of hens
Did not ovulate	2
Ovulated 1 ovum	4
Ovulated 2 ova	4
Ovulated 3 and 4 ova	2

both FSH and LH. The frequency of induced ovulations was very high, 10 of the 12 hens ovulating while none of the controls responded (Table 2). The two injected birds which did not ovulate were noted to be in poor physical condition at the time of autopsy. In this experiment the birds were killed 72 hours after the injection of the hormone and the number of ova shed was determined at autopsy (Huston and Nalbandov 1950).

TABLE 3. EFFECT OF INTRAVENOUS INJECTIONS OF LH (ARMOUR) ON OVULATIONS OF HENS WITH THREAD IN THE MAGNUM. (HENS INJECTED TWICE DAILY UNTIL FIRST OVULATION)

Treatment	Dose of LH (mgs.)	Number of hens not ovulating	Number of hens ovulating	Number of days from first injection to ovulation
Thread in magnum*	0.0	6	2	—
Thread in magnum*	0.1	1	6	2, 2, 3, 3, 4, 7
Thread in magnum*	0.2	3	5	2, 4, 5, 6, 8
Sham operated†	0.0	1	6	—

* Had not ovulated from operation to first injection (9 days).
† Were in normal production during the whole period.

Since six of the ten hens ovulating in response to the ISAP injection shed more than one yolk, the dosage used was thought to be unphysiologically high. In the second experiment LH (Armour) was injected at two different dosages into two groups of hens of seven and eight animals respectively with threads in their magna. Both dosages of LH were effective in causing ovulations in the majority of the hens although the number of injections required to cause ovulations was much greater than in the case of ISAP (Table 3). The dosages of LH seemed within the physiological range since no more than one ovulation was produced in any of the treated hens.

It has been shown that release of LH from the pituitary can be induced in the fowl by progesterone (Fraps and Drury, 1943; Rothchild and Fraps 1949). Accordingly, the ability of progesterone to cause ovulation in hens with threads in the oviduct was assayed in a series of tests involving dosages of progesterone ranging from 0.3 to 6.0 mgs. In all cases a single

TABLE 4. ABILITY OF VARIOUS DOSES OF PROGESTERONE TO CAUSE OVULATION IN HENS WITH THREAD IN THE MAGNUM

Dose of progesterone (mgs.)	Number of hens not ovulating	Number of hens ovulating	Average number of ovulations per hen	Number of days from injection to ovulation
(oil)	6	0	0	—
0.3	3	0	0	—
0.6	3	0	0	—
0.9	3	0	0	—
1.0	3	0	0	—
2.0	2	2	1.00	2, 3
4.0	3	2	1.50	2, 5
5.0	3	4	1.25	1, 2, 2, 5
6.0	1	3	2.00	1, 2, 2

subcutaneous injection of the hormone was given. The results (Table 4) indicate that it is possible to cause ovulation with progesterone in birds in which the release of endogenous LH has been blocked by the presence of a loop of thread in the magnum. Apparently the progesterone levels required for ovulation in these hens are higher than those which had been found effective by Rothchild and Fraps (1949). These authors had established the egg-laying pattern of each hen accurately enough to predict within a few hours the expected time of maturation of a follicle. In the present experiments no such determination could be made, since laying had been interrupted by the placing of the irritant and most of the follicles of ovulatory size were quite old when the progesterone injections were made. The reduced effectiveness of progesterone observed in our experiments may be due to a loss of sensitivity of the "aged" follicles to LH or a reduced sensitivity of the pituitary to progesterone. This may explain the failure to induce ovulation in nine out of twenty hens injected with two or more milligrams of progesterone (Table 4).

During the course of these experiments it was noted that 20 to 40 days after the thread had been placed in the magnum, most of the operated birds gradually began to lay and eventually returned to normal laying frequency. This has been explained on the assumption that the neural mechanism involved becomes conditioned to the constant irritation of the oviduct and loses its ability to respond to the stimulus. To test this possibility two experiments were performed. In one group of eight birds laying was stopped by the insertion of a thread in the magnum. When the thread was removed, seven of the eight birds began to lay normally within two weeks after the operation.

A second group of eight birds, which had a thread in the magnum was allowed to go through the usual non-laying period following the operation. As in earlier experiments these hens began to lay 30 to 40 days after the operation. As they returned into production a second operation was performed, the original thread was removed and a new one placed in the same general region of the magnum. Six of the eight hens subjected to this treatment continued to lay indicating that the oviduct as a whole loses its sensitivity to respond to the stimulus from the thread.

DISCUSSION

On the basis of these experiments it is suggested that a neural link exists between the oviduct and the pituitary gland of chickens. This is supported by the fact that the presence of an irritant in the magnum suppresses ovulation without affecting the other endocrine functions of the pituitary, the ovary or the responses of their respective target organs. It is known that comb growth in the hen is caused by androgen and that androgen secretion in turn is caused by LH stimulation of the interstitial cells of the

ovary (Nalbandov, unpublished). Since in hens in which a thread was present in the magnum, comb-size remains unaffected (indicating continued secretion of *some* LH), the assumption becomes necessary that in the chicken ovulation is caused by the secretion of additional amounts of LH over and above that quantity of LH which is assumed to be produced at a constant rate. It is thought that the occurrence of these "ovulatory peaks" of LH are specifically prevented by the presence of an irritant in the magnum. This interpretation is supported by the observation that the injection of exogenous LH or of progesterone which releases endogenous LH from the pituitary, causes ovulation in hens with thread in the magnum, which stops as soon as injections are discontinued. This interpretation fits in with findings of Sawyer, Everett and Markee (1948, 1949) who showed that in the rat neurogenic activation of the pituitary is necessary for the release of LH. In the hen in which a thread is placed in the oviduct the neural pathway to the pituitary appears to be blocked.

It is of interest to note that in operated hens follicles which normally would have ovulated within a few hours, remained in a state of "suspended animation" over prolonged periods of time, in some cases for more than four weeks, without becoming atretic. Such follicles continued to secrete estrogen since the oviducts were maintained in size and retained their ability to secrete albumen. Furthermore, the follicles remained capable of ovulating over prolonged periods of time, as shown by the fact that ovulations resulted following injection of exogenous LH.

The interpretation presented fits most of the facts observed. There are certain findings which cannot be fitted into this scheme at the present time and which will require additional experimentation. Thus in some of the operated hens, particularly in the early experiments in which keychains and other large irritants were used, the ovary enlarged considerably, in some cases containing six or more follicles of ovulatory size (30 or more mm. in diameter). This was interpreted to mean that the rate of secretion of FSH and possibily LH was increased appreciably by the presence of the irritant but here too ovulation was suppressed. In some hens the presence of a thread in the magnum did not suppress ovulation but on the contrary caused superovulation. Thus in one case four eggs were laid in two successive days although all of them were soft-shelled. In many other cases two soft-shelled eggs were laid in one day.

The presence of a uterine irritant produces a very high proportion of either soft-shelled eggs or yolks coated only with albumen or even yolks which are laid completely uncoated. In one experiment eight normal hens laid 63 eggs, only one of which was soft-shelled while eight operated hens ovulated with LH produced 16 eggs, eight of which were either soft-shelled or consisted of yolks coated with albumen only. One of these hens laid two eggs in 12 hours, the first egg with a normal shell, the second soft-shelled.

These facts show that uterine irritation may occasionally cause an increased rate of both FSH and LH secretion leading to superovulation.

The existence of this hitherto unsuspected neurogenic connection between the oviduct and the pituitary gland raises the question whether this relationship has any physiological significance in the normal hen or whether it is an artifact. It is thought that the mechanism described is physiologically important in that it may serve the hen as a timing device which prevents ovulation while a yolk (an irritant) is passing through the oviduct. Additional work will be needed before the details of such a signalling system are understood and can be fitted into the time schedule already worked out with regard to the release of LH and the length of time an egg normally spends in the oviduct and in the shell gland.

SUMMARY

The presence of an irritant, such as a loop of thread, in the magnum portion of the oviduct of laying hens, completely suppresses ovulation in the great majority of the treated animals. There is no reduction in the size of the ovary, the oviduct or the comb of these hens for as long as 25 days.

Injection of progesterone or of gonadotrophin containing LH into such hens causes ovulations but only so long as the injections are continued.

The presence of an irritant is thought to prevent the secretion of amounts of LH sufficient to cause ovulation (ovulatory peaks), but does not interfere with the secretion of levels of LH sufficient to assure normal androgen secretion, as is evidenced by the fact that in hens with a thread in the oviduct the comb does not decrease in size. The presence of the loop does not prevent the secretion of normal amounts of FSH since the ovary remains of normal size for as long as 25 days after the operation. For approximately the same length of time the ovary also contains follicles of ovulatory size (30 mm. or over) and in the same numbers as would be expected in unoperated hens. These follicles continue to secrete estrogen at normal levels since the oviduct in operated hens does not decrease in size and remains capable of secreting albumen.

This neurogenic system may be of physiological importance in the normal hen in that it may serve to synchronize ovulation with the other events of the laying cycle.

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THE POTENCY OF CERTAIN DESOXYCORTICOSTERONE ESTERS WITH RESPECT TO THE PRODUCTION OF HYPERTENSION AND CARDIOVASCULAR LESIONS*

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OF THE 11-desoxycorticoids which are available for therapeutic use, desoxycorticosterone acetate (DC-acetate) has enjoyed the widest acceptance. In an effort to increase the activity of the hormone either by conferring upon the steroid a more favorable absorption rate or reducing the rate at which it is metabolized by the body, various esters, in addition to the commonly used acetate, have been prepared. The water soluble DC-glucoside has proven to be very effective, when administered intravenously, in effecting the recovery of adrenalectomized dogs from the symptoms of acute adrenal insufficiency (Meier *et al.*, 1944; Swingle *et al.*, 1952). DC-benzoate, DC-palmitate and DC-butyrate have each been found, when given in identical dosage by single injections, to prolong the life of adrenalectomized rats more effectively than either dexoxycorticosterone or its acetate.

With the recent preparation of DC-trimethylacetate and DC-phenylacetate, attention has again been focused on the metabolic effects of desoxycorticosterone esters. Both of the latter compounds have been found to be more effective than the acetate when given as single injections, in maintaining the adrenalectomized dog free of insufficiency symptoms (Swingle *et al.*, 1952; Gross and Tschopp, 1952), or in prolonging the survival period of adrenalectomized rats (Gaunt *et al.*, 1952; Gross and Tschopp, 1952). Clinically, the trimethylacetate has been found, again administered as a single injection, to have prolonged effects in patients with Addison's disease and acute effects of overdosage have not been observed (Frawley and Forsham, 1951).

There is no reason *a priori* to suppose that the different esters of desoxycorticosterone would vary qualitatively in their actions, although quantitatively dissimilar activities might be anticipated. In view of the fact that toxic symptoms of overdosage with DC-acetate, including hypertension, edema and cardiac failure have been observed in man (Giraud

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et al., 1950; Willson *et al.*, 1940; Thorn *et al.*, 1940) and the rat (Selye and Hall, 1944; Friedman *et al.*, 1948), it might be expected that other desoxycorticosterone esters would display similar effects. Because the studies on DC-phenylacetate and DC-trimethylacetate reported to date appear to have been concerned exclusively with the effects of a single injection, an experiment designed to test the effect of chronic treatment with desoxycorticosterone esters in order to assess their relative toxicity with respect to the cardiovascular system appeared to be warranted.

METHODS

Forty female albino rats of the Holtzman strain weighing between 60 and 89 gms. with an average of 75 gms., were uninephrectomized and divided into four equal groups. The rats of groups 1, 2, and 3, each received 2 mg./day of DC-acetate,† DC-phenylacetate and DC-trimethylacetate respectively, by subcutaneous injection, in 0.2 cc. of the suspension medium. The animals of group 4 each received 0.2 cc./day of suspension medium and served as controls. All animals were maintained on a regimen of Purina Laboratory Chow and 0.9% NaCl solution *ad lib.*

Blood pressures, on unanaesthetized animals, were measured on the first day and once weekly thereafter until the experiment was terminated. A modification (Sobin, 1946) of the tail plethysmograph method (Williams *et al.*, 1939) was employed, and the arithmetic mean of four consecutive pressures agreeing to within 5 mm. Hg was taken to be representative of the blood pressure of the rat in question. The daily fluid intake of each of the groups of experimental animals was measured. The animals were sacrificed on the 28th day of the experiment, and the various tissues and organs were excised and placed in Bouin's solution for fixation and subsequent histologic treatment. After fixation, the organs to be weighed were transferred to water for 24 hours. They were then carefully dissected free of connective tissue, blotted on filter paper and weighed on a torsion balance.

RESULTS

Fluid Intake

The fluid intake of each of the four groups is given in Figure 1. It is apparent that the daily consumption of fluid was greatly augmented in each of the steroid treated groups, although those receiving DC-acetate showed the greatest evidence of polydipsia, and those receiving DC-trimethylacetate the least. Until the third week of treatment, at which time hypertension became more marked in the DC-trimethylacetate group, these animals were consuming about twice as much of the saline solution as controls, while those receiving DC-acetate consumed about three times as much. The fluid intake of rats receiving DC-phenylacetate, while slightly less than that of the DC-acetate group, paralleled the latter closely throughout the major part of the experiment.

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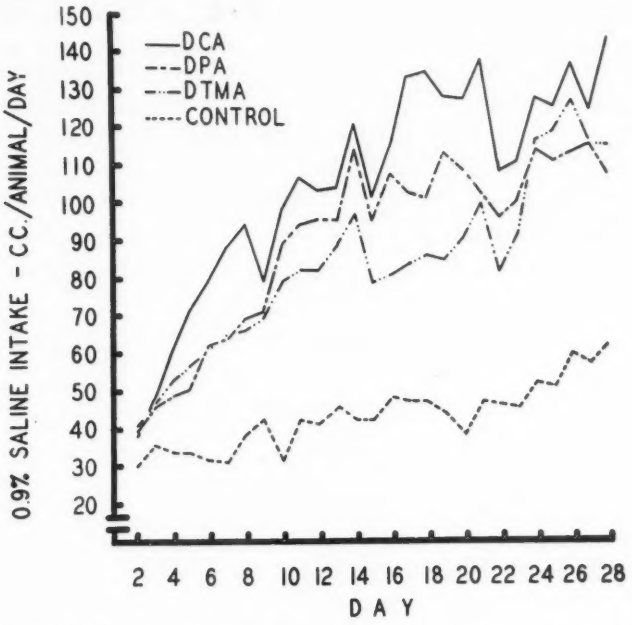

FIG. 1. Intake of 0.9% NaCl solution in control rats and rats receiving the acetate, phenylacetate and trimethylacetate esters of desoxycorticosterone.

Blood Pressure

In the various studies on hypertension which we have conducted in the rat, rarely is a pressure of 135 mm. Hg or higher encountered in unanesthetized normal animals from our colony. However, since an occasional rat may show pressures of 145 mm. Hg, a level of 150 mm. Hg has arbitrarily been selected as the lowest pressure consistent with a diagnosis of hypertension. While it is possible that a few animals having pressures which lie between 140 and 150 mm. Hg might be truly hypertensive while not considered as such, at least the level of 150 mm. Hg permits us to be more certain that such a pressure has resulted only through treatment.

No evidence of hypertension was encountered at any time in the control animals of group 4. The behavior of groups 1 and 2, receiving the acetate and phenylacetate respectively was essentially similar. There was early onset of hypertension, detected in the second week of treatment,which became progressively more severe, with the result that on the 26th day of treatment all of the animsls in these groups were hypertensive, the group average being in excess of 210 mm. Hg. The animals of group 3, receiving trimethylacetate, were much less affected. While there was an

early onset of hypertension in four of the animals, i.e. on the 7th day, the incidence did not increase until the fourth week of treatment in which, for the first time, the average blood pressure of the group exceeded 150 mm. Hg. At the last blood pressure determination, the group average was less than 190 mm. Hg and two of the nine survivors were normotensive, having pressures below 140 mm. Hg. Hypertension in group 3 tended therefore, to be later in onset, milder in severity and of lesser incidence than in groups 1 and 2. Table 1 summarizes the data.

TABLE 1. EFFECT OF DESOXYCORTICOSTERONE ESTERS ON BLOOD PRESSURE OF THE RAT

Day	Blood pressure	Controls (group 4)	Desoxycorticosterone		
			Acetate (group 1)	Phenyl-acetate (group 2)	Trimethyl-acetate (group 3)
1	No. hypertensive	0	0	0	0
	Group average, mm. Hg.	112 ± 2†	112 ± 2	111 ± 2	115 ± 3
7	No. hypertensive	0	4	1	4
	Group average, mm. Hg.	111 ± 2	142 ± 6	135 ± 4	148 ± 4
14	No. hypertensive	0	5	7	3
	Group average, mm. Hg.	115 ± 3	158 ± 11	156 ± 4	143 ± 6
21	No. hypertensive	0	9	10	4
	Group average, mm. Hg.	115 ± 2	176 ± 11	193 ± 7	147 ± 10
26*	No. hypertensive	0	9	9	7
	Group average, mm. Hg.	119 ± 2	218 ± 11	214 ± 10	183 ± 12

* Nine animals surviving in each steroid treated group on this day.
† Mean ± S.E.

Miscellaneous Observations

The animals of groups 1 and 2, which showed early onset of severe hypertension and marked polydipsia also developed edema and ascites during the second week of treatment and continued to manifest these changes in varying degree throughout the period of treatment. The animals of group 3, treated with DC-trimethylacetate, increased their fluid intake markedly, i.e. from about 90 cc./rat/day to about 125 cc./rat/day during the last week of treatment, at this time they also began to develop the edema and ascites which had an earlier onset in groups 1 and 2, and an additional three animals became hypertensive.

Retinal hemorrhages, a not uncommon symptom of hypertensive oculopathy in DCA treated rats, was observed in two animals in group 1 and one animal in group 2. They did not occur in groups 3 and 4.

Organ Weights: These data are given in Table 2.

Adrenals. There was no significant alteration in adrenal weight as a result of treatment with the DC-esters. This is in conformity with published

observations on the effect of DC-acetate. Since the main effect of 11-desoxycorticoids is to cause involution of the cortical zona glomerulosa, which constitutes an insignificant proportion of the total bulk of the gland, changes in adrenal weight are never pronounced in animals treated with these hormones.

Kidneys. The renal enlargement, so characteristic of DCA treated rats, was equally marked in rats treated with DC-phenylacetate and DC-

TABLE 2. ORGAN WEIGHTS AND CARDIOVASCULAR LESIONS IN CONTROL AND STEROID TREATED RATS

Item	Controls	Desoxycorticosterone		
		Acetate	Phenylacetate	Trimethyl-acetate
Body wt. g. Initial Final	 73 ± 3 146 ± 17	 75 ± 3 141 ± 5	 75 ± 4 122 ± 6	 75 ± 3 141 ± 4
Adrenal mg./100 g.	24.6 ± 1.5	22.0 ± 0.9	29.0 ± 2.0	23.7 ± 1.4
Heart mg./100 g. Incidence and severity of lesions	298 ± 14 0 (10)	449 ± 43 + (3) * ++ (1) +++ (2) 0 (3)	453 ± 29 + (2) ++ (3) +++ (3) 0 (1)	378 ± 27 + (4) ++ (1) +++ (0) 0 (4)
Kidney mg./100 g. Incidence and severity of lesions	621 ± 15 0 (10)	1307 ± 89 + (3) ++ (4) +++ (2) 0 (0)	1360 ± 96 + (2) ++ (3) +++ (4) 0 (0)	1118 ± 104 + (2) ++ (3) +++ (2) 0 (2)
Pancreas Incidence and severity of lesions	0 (10)	+ (5) ++ (1) +++ (0) 0 (3)	+ (3) ++ (2) +++ (3) 0 (1)	+ (0) ++ (3) +++ (0) 0 (6)

* Severity, designated by pluses, is followed by bracketed figure indicating incidence.

trimethylacetate; there being no significant difference in renal weight between any of the steroid treated groups.

Heart. Cardiac hypertrophy was evident in each of the steroid treated groups, although not as marked in the group which received DC-trimethylacetate as in those treated with the acetate or phenylacetate.

Cardiovascular Lesions

Histologic examination of the kidney, heart and pancreas, the latter having proven to be a site of predilection for the necrotizing arteriolitis which occurs in hypertensive DCA treated rats, revealed that typical lesions were present in animals in each of the groups. However they were of lower incidence and lesser severity in the trimethylacetate than in the acetate or phenylacetate treated rats. A summary given in Table 2 synop-

sizes the data. The severity of lesions are graded on a scale of 0 to +++. In the pancreas the lesions were entirely vascular, of the periarteritic type; in the heart they consisted of either arteriolar necrosis with an associated perivascular accumulation of inflammatory cells, resembling the "Aschoff nodule"; or as an interstitial myocarditis. The renal lesions were those of arteriolosclerosis and necrosis, glomerulosclerosis and occasionally focal infiltrations of inflammatory cells. The incidence of lesions in each of these tissues was approximately equal in the acetate and phenylacetate groups and less in the trimethylacetate treated rats.

DISCUSSION

Insofar as polydipsia and the development of hypertension may serve as criteria in the estimation of mineralocorticoid toxicity, these experiments suggest that DC-acetate and DC-phenylacetate are of approximately equal potency whereas DC-trimethylacetate is somewhat weaker. The conclusion is also supported by the incidence and severity of cardiovascular lesions. Although, it would be hazardous to draw sweeping conclusions from one experiment involving so few animals, in view of the variability of such responses (Rosenberg *et al.*, 1952), the present experiment suggests that DC-phenylacetate is somewhat more toxic than DC-acetate.

The effects of DC-trimethylacetate, as compared with those of the acetate and phenylacetate derivatives, were relatively delayed in onset. This was manifest in saline intake and the development of hypertension. Since we have never observed lesions of the type described in desoxycorticosterone-treated rats unless hypertension was present, and since the severity of the lesions is correlated with the duration and severity of hypertension, it was not surprising that the cardiovascular lesions were of lower incidence and lesser severity in the DC-trimethylacetate group.

The conclusion which appears to be best compatible with the results obtained is that each of the esters is capable of producing hypertension and cardiovascular damage, but that DC-trimethylacetate, possibily because of a slower absorption rate, requires a longer period of time to exhibit the same toxic potentiality as the acetate and phenylacetate.

Since the evidence previously cited indicates that at a given dosage DC-trimethylacetate exerts a more prolonged effect than the acetate, and inasmuch as it is more effective than DCA even at a lower dosage in supporting life and growth in the adrenalectomized rat (Gaunt *et al.*, 1952), it would seem likely that DC-trimethylacetate will prove, because of its lower toxicity, to be preferable to the acetate in controlling the electrolyte imbalance in patients.

SUMMARY

A comparison of the toxicity of three desoxycorticosterone esters, namely the acetate, phenylacetate and trimethylacetate, has been made in the rat. The criteria used were the induction of hypertension and cardiovascular lesions in the uninephrectomized rat maintained on a high NaCl intake and receiving 2 mg./day of the steroid subcutaneously.

The results indicate that the onset of hypertension, severe polydipsia, edema and ascites is rapid in rats receiving either the acetate or phenylacetate and delayed in rats treated with the trimethylacetate ester.

Cardiovascular lesions, while present in all groups were of lowest incidence and least severity in rats treated with DC-trimethylacetate.

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A QUANTITATIVE STUDY OF THE VASCULARITY OF THE ADRENAL GLAND OF THE RAT DURING PREGNANCY AND AFTER CORTISONE TREATMENT[1,2]

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THE maintenance and activity of cells of an organ is dependent among other things on the functional adequacy of the blood supply. The functional adequacy of the circulation of any organ depends in turn on a number of factors: the blood volume, rate of blood flow, the concentration of gases, nutrients and metabolites in the blood and tissue fluid, the tissue permeability of these substances, and the area of the capillary bed. In an endocrine organ, like the adrenal cortex, these factors are especially important, as the functional activity is expressed by the pattern of blood circulation. The complexities in the morphology of the adrenal cortex of mammals are so great that it seems practical at the present time to approach experimentally only the estimation of the area of the capillary bed. This area may be expressed as a ratio of the surface of the capillary bed to the volume of tissue occupied by it (S. V.).

There are no quantitative estimations of the vascularity in the adrenal gland despite several good qualitative approximations (Sjöstrand, 1934; Gersh and Grollman, 1941; Bennett and Kilham, 1940). The difficulties in the way of a quantitative estimation are apparent. It was hoped that many of these difficulties, if not most, could be avoided by the use of fixation by freezing and drying. Such a study has been made by Gersh and Still (1945) for fat tissue, and essentially the same technique was used in this study.

The experiments were undertaken in normal female and pregnant rats, with and without cortisone treatment. After very rapid fixation of the adrenals in situ by freezing with flowing isopentane at about −150° C., blocks of tissue were removed and dried at a low temperature. They were

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embedded in celloidin and sectioned serially. Camera lucida drawings of the outlines of the capillaries containing red blood cells were made. From the drawings were obtained data that gave the ratio of the total capillary surface to the volume of tissue supplied by the functioning vessels (S/V ratio), and the mean diameters of the capillaries.

It was found that marked changes of the S/V ratio and of the mean diameter of the capillaries occurred in the zona fasciculata and in the zona reticularis during pregnancy and after cortisone acetate treatment. In marked contrast is the relative constancy of the vessels in the zona glomerulosa. The changes in the medullary capillaries, if significant, were small.

MATERIAL AND METHODS

Preparatory phase. Twelve female rats of the Sprague-Dawley strain were used. They were fed "Complete Rockland Rat Diet" and they were allowed water *ad libitum*. Three

TABLE 1

No.		Weight at the beginning, gms.	Weight after 18–19 days, gms.
1		192	221
2	Controls	196	219
3		188	217
4	Non-pregnant rats. Daily 3 mg.	183	148
5	Cortisone acetate	196	165
6		182	156
7		187	280
8	Pregnant rats	185	269
9		188	267
10	Pregnant rats 3 mg. Cortisone	193	240
11	acetate daily	196	251
12		201	251

Summary of the weights of the animals at the beginning and at the end of the preparatory phase.

normal rats served as controls; 3 non-pregnant rats were treated with 3 mg. cortisone acetate daily for 19 days. Six animals in proestrus were caged with males in the afternoon and when sperm were identified in the vaginal smear on the following morning this was considered as day one of pregnancy. Three of these rats were treated with 3 mg. cortisone acetate daily for 18 days; the other 3 received no treatment. Table 1 presents the summary of the weights of the animals at the beginning and at the end of the preparatory phase.

Preparation of the adrenals. Under light ether anesthesia the abdominal cavity was rapidly opened, the left adrenal exposed and immediately frozen in situ by a stream of isopentane chilled to about −150° C. The region including the frozen adrenal was then broken out with a chilled bone forceps. The gland was transferred to the drying chamber, and dried at −25° C. for eight days (Gersh, 1932). The adrenals were then placed in absolute alcohol at −25° C., and after 24 hours allowed to warm up to room temperature.

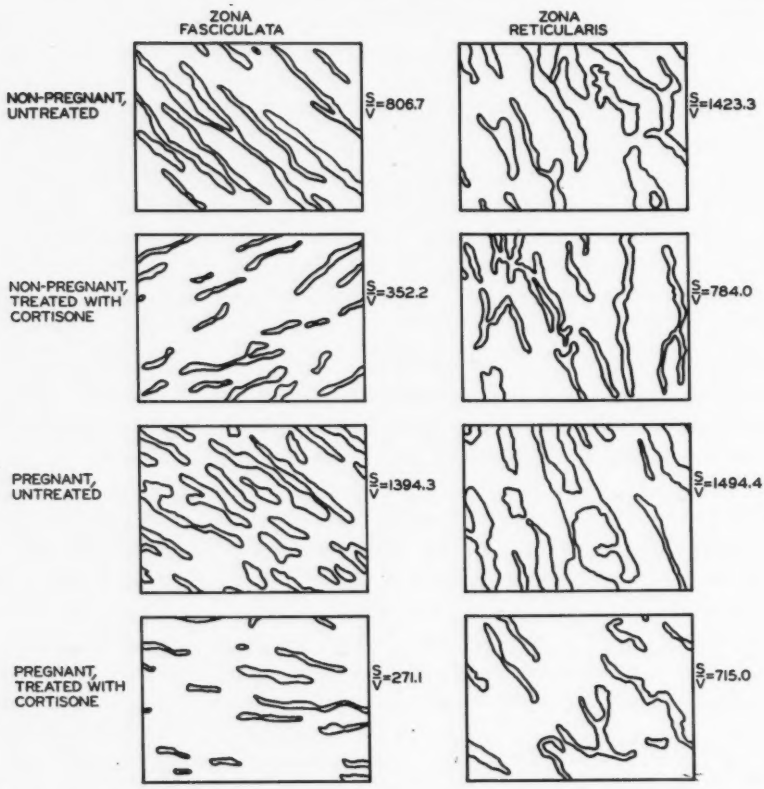

FIG. 1. Sample Camera Lucida drawings of portion of sections of the adrenal cortex to show differences in diameter and surface area of capillaries of non-pregnant and pregnant rats untreated and treated with cortisone.

The adrenal glands were then shelled out and embedded in celloidin. Serial sections 10 microns thick were cut and mounted on slides without staining.

Measurements of sections. Camera lucida drawings were made of the outline of capillaries containing red blood cells in 15–20 fields selected at random of sections through the largest diameter of the gland (Fig. 1). The surface of each capillary was measured with a planimeter, as well as the area of the whole field which was drawn. The mean diameter of the capillaries was determined by 150–200 measurements in each group. The thickness of each zone of the adrenal gland was measured with a calibrated ocular micrometer.

Calculations. The ratio of the surface of the capillary bed to the volume of tissue supplied by the vessels (S/V) was calculated by the formula used by Gersh and Still (1945).

$$S/V = \frac{1.4142\ (\text{Total area of capillaries on drawing})}{(\text{Area of section}) \qquad (\text{Thickness of section})}$$

The true diameter of the capillaries was determined by division of the mean diameter on

the drawings by the magnification. For quantitative estimations it appeared justified to assume the adrenal to be sphere. The volumes of the spherical layers of the different zones were obtained by subtracting appropriate spherical layers, i.e.

$$\text{Volume (Glomerulosa)} = \frac{4\pi r^3}{3} - \frac{4\pi(W_f + W_r + W_m)^3}{3}$$

r = radius of the adrenal
W_f = width of the fasciculata
W_r = width of the reticularis
W_m = width of the medulla

The true total surface of the capillary bed of each zone was determined by multiplication of the appropriate S/V ratios to the volume of the zones.

The magnitude and tediousness of the labor involved necessitated the use of a relatively small number of experimental animals. Only large differences were considered to be of significance.

RESULTS

Zona glomerulosa. There were no changes in the vascularity of the zona glomerulosa under the different experimental conditions. In spite of the

TABLE 2

		Controls	3 mg. cortisone acetate daily	Pregnant rats	Pregnant rats 3 mg. cortisone acetate daily
Zona glomerulosa	Mean diameter of the capillaries	4.1	3.9	4.2	4.1
	S/V ratio (cm^{-1})	344.9	379.9	312.6	340.0
Zona fasciculata	Mean diameter of the capillaries	6.1	3.6	7.5	3.6
	S/V ratio (cm^{-1})	805.2	368.0	1297.1	246.6
Zona reticularis	Mean diameter of the capillaries	9.3	6.4	10.0	6.7
	S/V ratio (cm^{-1})	1172.9	837.0	1483.0	592.5
Medulla	Mean diameter of the capillaries	8.5	6.2	7.5	6.0
	S/V ratio (cm^{-1})	294.9	247.9	272.5	226.7

The S/V ratio and mean diameters of the capillaries under different experimental conditions. Marked changes in the surface of functioning capillaries in the zona fasciculata and zona reticularis are in contrast with relative constancy of the vessels in the zona glomerulosa. The changes in the capillaries of the medulla do not seem to be significant.

decrease of the total volume of the gland after cortisone acetate treatment the glomerular zone increased in width. This increase reflects the maintenance of the volume of the zone on the surface of the shrinking sphere.

Zona fasciculata. A large increase of the S/V ratio occurred during pregnancy (see Table 2). The mean diameter of the capillaries was also increased during pregnancy (6.1 microns for normal rats, 7.5 microns for pregnant rats). After cortisone acetate treatment of the non-pregnant rats and of

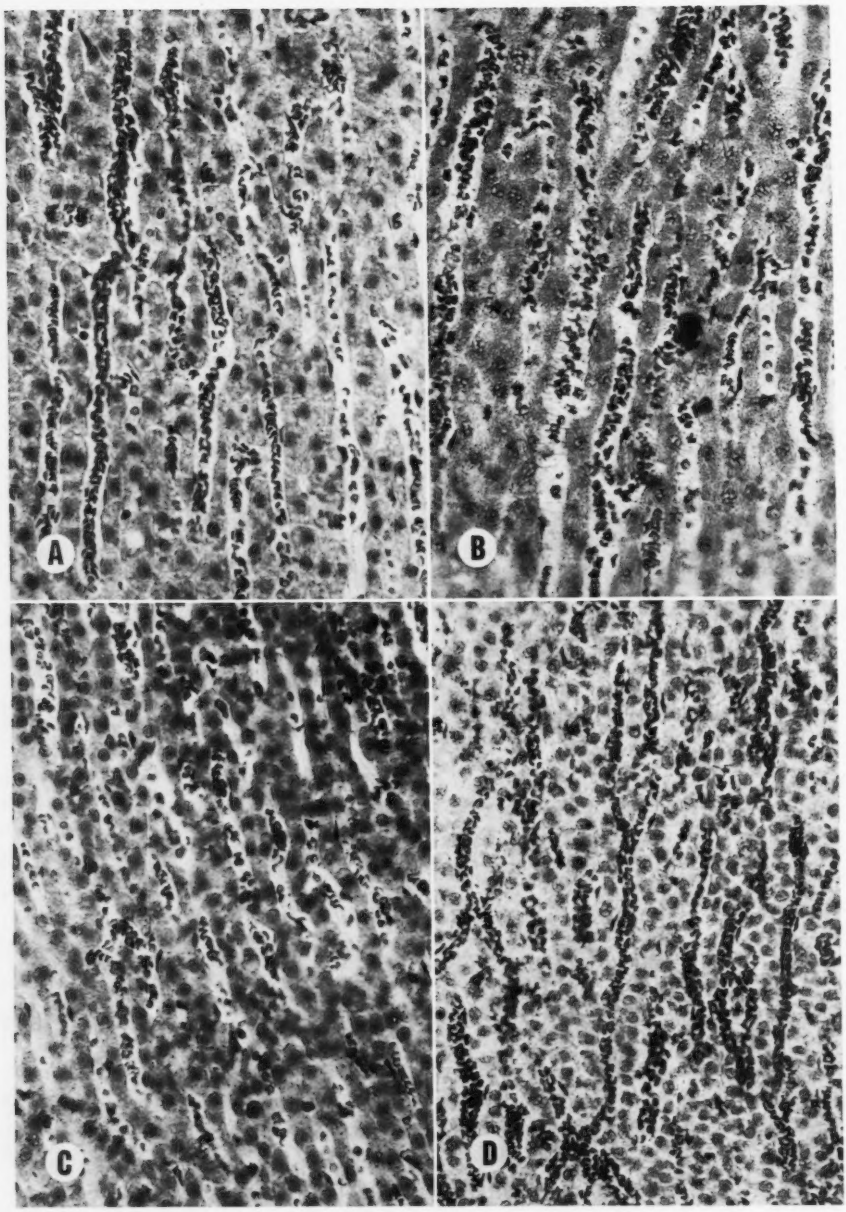

PLATE I

TABLE 3

		Controls	3 mg. cortisone acetate daily	Pregnant rats	Pregnant rats 3 mg. cortisone acetate daily
Zona glomerulosa	Width	68.7	90.0	64.2	107.3
	Volume mm.^{3}	3.4	2.8	3.3	3.1
	Total capillary surface mm.^{2}	1176	1056	1003	1062
Zona fasciculata	Width	833.6	529.0	817.0	585.0
	Volume mm.^{3}	25.8	9.1	24.6	10.4
	Total capillary surface mm.^{2}	20744	3365	31857	2552
Zona reticularis	Width	491.8	242.7	370.5	283.5
	Volume mm.^{3}	4.5	1.8	3.9	2.0
	Total capillary surface mm.^{2}	5115	1523	5846	1168
Medulla	Width	703.3	656.9	737.5	597.5
	Volume mm.^{3}	1.6	1.2	2.2	0.9

The width, volume and total capillary surface of the different zones under the different experimental conditions. Marked changes in the total capillary surface in the zona fasciculata and zona reticularis are in contrast to the relative constancy of the vessels in the zona glomerulosa. The total surface of functioning capillaries of the medulla was not determined because measurements of the medullary sinusoids and veins were not made.

the pregnant rats the S/V ratio was significantly lowered and the mean diameter of the capillaries was reduced below that of untreated controls (3.6 microns for both treated groups) (Fig. 2 A, B, C, D.).

These differences in the surface area of capillaries per unit volume are magnified when one takes into account changes in the volume of the fascicular zone (see Table 3). The total capillary surface of the zone showed an increase from 20,747 mm.^{2} to 31,857 mm.^{2} in pregnancy and a decrease to 3,365 mm.^{2} and 2,552 mm.^{2}, respectively after cortisone treatment.

Zona reticularis. There was a definite increase of the S/V ratio during pregnancy and possibly an increase of the mean diameter of the capillaries

←

EXPLANATION OF PLATE I

FIG. 2. Photomicrographs to illustrate changes in the diameter and in the number of open capillaries of the zona fasciculata of the rat are more numerous and their lumens are wider in diameter than in the non-pregnant rat. After treatment with cortisone the diameter and the number of open capillaries were markedly reduced in both pregnant and non-pregnant animals. The adrenals were prepared by freezing and drying, embedding in celoidin, and staining with hematoxylin eosin (×550).

A. Normal, non-pregnant. B. Normal, pregnant. C. Non-pregnant, treated with cortisone, and D, pregnant, treated with cortisone.

(from 9.3 microns for normals to 10.0 microns for pregnant animals). (See Table 2.) After cortisone treatment of non-pregnant and pregnant rats the S/V ratios were reduced below figures obtained in the untreated controls. The mean diameter in the non-pregnant group was reduced to 6.4 microns and in the pregnant group to 6.7 microns after cortisone administration.

The total capillary surface in the zona reticularis was increased during pregnancy (5,115 mm.^{2} for normal rats, and 5,846 mm.^{2} for pregnant rats). Cortisone acetate treatment caused a definite decrease of the total capillary surface of the reticular zone in non-pregnant rats (1,523 mm.^{2}) as well as in pregnant animals (1,168 mm.^{2}).

Medulla. A slight decrease of the S/V ratio which may be not significant was observed after cortisone treatment. The mean diameter of the capillaries was reduced from 8.5 microns (non-pregnant) and 7.5 microns (pregnant) to 6.2 microns and 6.0 microns, respectively; whether these changes are significant is also not known. No changes occurred in the pregnant rats. The total capillary surface was not determined in the medulla because measurements of the medullary sinusoids and veins were not taken. As these constitute a significant proportion of the total medullary volume the values of the total capillary surface in the absolute volume of the medulla would be misleading.

DISCUSSION

The very rapid fixation of the adrenals in situ by freezing at about −150° C. makes it possible to determine approximately the dimensions of the capillaries containing red blood cells at the moment of freezing (Gersh and Still, 1945). The knowledge of the so obtained S/V ratio of the different zones of the adrenal contributes to the understanding of the functional activity of the gland.

In contrast to findings in other species several investigators (Donaldson, 1924, 1928; Anderson and Kennedy, 1933; Blumenfeld, 1934) did not find a significant increase in adrenal weight during pregnancy. However, a definite increase of the S/V ratio and of the total capillary bed of the zona fasciculata and zona reticularis was observed during pregnancy in these experiments. The S/V ratio for the zona reticularis of the pregnant rats (1,483) is the highest S/V value ever reported for tissue. Catchpole and Gersh (1947) calculated for the medulla of the mouse kidney 1,400, for the kidney cortex 850, for the heart muscle of man 1,184, for the mouse liver, 800. The values for other tissues (skeletal muscle, brain cortex, cerebellum, duodenum, pancreas) range between 190 and 500.

Severe adrenal atrophy in rats has been described after high dosages of cortisone (Ingle and Mason, 1938; Wells and Kendall, 1940; Stebbins,

1950; Winter *et al.*, 1950) due to reduction in size of the cells of the fascicular and reticular zones. A complete loss of lipid from the cells of these zones has been observed. Sourkes and Henage (1951) stated that adrenals of cortisone treated rats showed a decreased endogenous respiration as well as greatly reduced rates of oxidation of various intermediates of the Krebs' cycle. While the total volumes of the zona fasciculata and zona reticularis were reduced to nearly one third of the original volumes after cortisone acetate treatment, the total capillary surface of these zones was reduced to approximately one fifth to one tenth of the capillary bed of the controls and of the pregnant adrenals.

Sjöstrand (1934) stated that the same factors which are responsible for excessive cortical activity are effective on the vascular pattern of the medulla. Gersh and Grollman (1941) reported that the same vascular adjustments appear to take place in the medulla during hyperactivity as were described in the cortex, though to a less marked degree. It seems that no change takes place in the medulla of the adrenals of pregnant rats. The decrease in the S/V ratio and in the mean diameter of the capillaries was so small after cortisone treatment that these changes may be considered also as not significant.

SUMMARY

The ratio of the surface of the capillaries to the volume of tissue supplied by vessels (S/V ratio) of the zona fasciculata was markedly increased in pregnancy, and reduced below normal levels after the daily administration of 3 mg. cortisone acetate for a period of 18–19 days. Similar changes of the S/V ratio were obtained in the zona reticularis.

These differences in the surface area of capillaries became magnified by taking into account the volume of the zones. The total capillary surface of the zona fasciculata was increased during pregnancy by one half. It was reduced by one fifth to one tenth after cortisone acetate treatment.

Changes of the vascularity of the zona glomerulosa were not observed under the different experimental conditions. The S/V ratio in the medulla was not altered markedly in the pregnant animals and the slight decrease of the S/V values after cortisone treatment was too small to be considered significant.

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EFFECTS OF TESTOSTERONE PROPIONATE ON HISTOCHEMICAL REACTIONS OF RAT SEMINAL VESICLES

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INTRODUCTION

THE English anatomist, John Hunter (1786), was the first to describe the seminal vesicles of the rat. His description is as follows: "the bags are large and flat with serrated edges and lie someway within the abdomen, containing a thick ash coloured mucus, nearly the consistency of soft cheese. . . . " Nearly 150 years later Moore, Hughes, and Gallagher (1930) made a detailed cytological study of this organ and demonstrated the fundamental relationship existing between the testicular hormone and the functional activity of the secretory epithelium. Recently Porter and Melampy (1952) have presented the results of a biochemical study of the composition and metabolism of this gland.

This investigation deals with the effects of testosterone propionate on the structure and function of the seminal vesicles of the rat. Histochemical observations were made on basophilia, the localization of alkaline and acid phosphatases, and periodic acid-reactive carbohydrates. Furthermore, information is presented on the distribution of connective tissue in the gland as well as mitotic activity. Intact, castrate, and hormone-treated castrate animals were used in this work.

MATERIALS AND METHODS

Histochemical changes in the seminal vesicles of the rat were studied in 3 groups of animals: (1) 6 intact sexually mature males, (2) 19 castrates, 2, 5, 10 and 20 days after gonadectomy, and (3) 23 castrates receiving subcutaneously 500 μg. of testosterone propionate (T.P.)† daily for 2, 5, 10, and 20 days. The seminal vesicles of the animals in group 3 were allowed to atrophy for a 20-day period following castration prior to hormone treatment. The animals in groups 2 and 3 were mature males weighing 240–255

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† Peranderen, Ciba Pharmaceutical Products, Inc., Summit, N. J.

gm. at time of castration. The rats were of the Holtzman-Rolfsmeyer strain and were fed Purina Laboratory Chow supplemented with raw carrots.

The following fixatives, reactions and staining techniques were used in this investigation.

Basophilia. A modification of the toluidine blue procedure of Kamell and Atkinson (1948) for ribonucleic acid was used. In this study the staining was carried out in a 0.05% solution of toluidine blue in a citrate-phosphate buffer (pH 5.0) for 30 minutes at 37° C. Control determinations for this method were made by incubating adjacent sections for 3 hours at 56° C. in a 0.1% solution of ribonuclease in veronal buffer (pH 6.7) as described by Herman (1950). Following enzyme treatment, the slides were washed and stained as for untreated sections. The eosin-methylene blue procedure was also used as described by Herman (1950) following fixation in Zenker's solution. Control preparations for this method were prepared by incubating sections for 3 hours in a 0.2% ribonuclease solution in citrate-phosphate buffer pH 7.0.

Alkaline and acid phosphatases. Seminal vesicle tissue was fixed in acetone for 24 hours at approximately 4° C. Following fixation, the tissues were dehydrated in two changes of acetone at room temperature for 3 hours, cleared in benzene and embedded in paraffin at 56° C. Sections in paraffin ribbons were incubated directly in the substrate according to Goetsch, Reynolds, and Bunting (1952). Alkaline phosphatase activity was determined at pH9 in α-sodium glycerophosphate substrate (Gomori, 1939) and the incubation time was 2 hours. The reagents used in the acid phosphatase reaction (pH 5) were prepared according to Gomori (1950) and α-sodium glycerophosphate was used as a substrate. The incubation times were 15 and 30 minutes as well as 1, 2, 3, 4, and 5 hours. In some cases localization of enzyme activity was also carried out on sections deparaffinized prior to incubation. Air-dried smears of seminal vesicle secretion were prepared and fixed in acetone as described for tissue.

Connective tissue. Zenker's solution was used as a fixative and Krichesky's (1931) modification of Mallory's triple stain was employed.

Periodic acid-reactive carbohydrates. Seminal vesicle tissue of intact rats was fixed in Orth's fluid for 2 days, washed overnight with tap-water, dehydrated in dioxan and embedded in paraffin. The method of staining was that of McManus (1946) using the time schedule suggested by Herman (1950). Two types of controls were used as recommended by Leblond (1950). These consisted of: (1) sections stained without previous passage through periodic acid, and (2) sections treated with salivary amylase for 1 hour at 37° C. in order to remove polysaccharides.

Colchicinic mitoses. Secretion-free glands from intact as well as 1-, 2- and 20-day T.P. injected castrate rats were fixed in Bouin's picro-formal. Six hours prior to killing, the animals were injected subcutaneously with colchicine dissolved in water at the rate of 0.1 mg. per 100 gm. body weight as suggested by Burkhart (1942). Sections were cut at 10μ, stained with Delafield's hematoxylin and eosin Y, and counts were made of mitotically dividing cells.

RESULTS

Basophilia. A strong basophilic reaction was obtained with toluidine blue in the secretory epithelium of the seminal vesicles of intact as well as hormone injected castrates, and was no longer obtained after ribonuclease action. Reactions in the seminal vesicles of an intact animal are shown in Figures 1 and 2. The nuclei of the epithelium, connective tissue, and

smooth muscle retained their blue coloration after enzyme hydrolysis. The staining response of the nucleoli of epithelial nuclei was not uniform with toluidine blue following ribonuclease action and a similar variability was also observed with eosin-methylene blue. In some cases the nucleolus was surrounded by a ring of basophilic material which was not acted upon by the enzyme. Although the secretory epithelium had atrophied in the 20-day castrate rat, as light basophilic reaction was observed in the scant cytoplasm of these cells. Upon treatment with ribonuclease, this cytoplasmic reaction was lost. In sections stained with toluidine blue there was no evidence of metachromasia in the seminal vesicles of intact, castrate, and hormone-treated animals with the exception of a few mast cells located in the fibro-muscular layers. Wislocki (1949) has previously reported metachromatic staining of mast cells of the stroma of seminal vesicles of deer.

A basophilic reaction was also noted in the cytoplasm of the secretory epithelium of the seminal vesicles of intact males following staining with eosin-methylene blue (Fig. 3). This reaction was abolished by ribonuclease. However, the secretory granules scattered throughout the apical cytoplasm were stained a brilliant pink and the halos surrounding them appeared lightly stained.

The cytoplasmic basophilia of the epithelium of the seminal vesicles of the rat was retained as the cells transformed from the secretory columnar type to the cuboidal after castration (Fig. 4). Following 2 days of T. P. treatment, the secretory epithelium commenced to show the effects of the hormone and a basophilic reaction was evident in the cytoplasm but secretion granules were absent. After 5 days of hormone injection the seminal vesicles gave the cytological appearance of having almost recovered from the effects of castration. The secretory epithelium had become the stratified columnar type and exhibited a basophilic reaction in the cytoplasm but only a few red staining secretory granules were observed at the apex of some of the epithelial cells. The seminal vesicles presented the histological characteristics of a normal gland after 10 days of hormone treatment.

In all intact, castrate, as well as hormone-treated animals the nuclei, and their nucleoli, of the secretory epithelium showed a strong basophilia when stained with eosin-methylene blue. This nuclear basophilic reaction, excepting that of the nucleolus, was not affected by ribonuclease. The staining reaction of the nucleolus after enzyme treatment was somewhat variable. In some cases the nucleoli disappeared completely and in others the whole structure retained its basophilic reaction. As suggested by Danielli (1946) it is possible that the results obtained in this investigation may be due to the fact that the nucleic acid might be bound or covered in such a manner than the ribonuclease could not reach its substrate. Other possi-

ble sources of error in the use of purified ribonuclease in cytochemical studies have been discussed by Stowell and Zorzoli (1947).

The lamina propria contained methylene blue-staining cells, pink fibers, and brightly stained erythrocytes and no basophilia was observed in the arterioles and venules. The secreted material present in the lumen was stained with eosin.

Alkaline and acid phosphatases. The results obtained by a histochemical investigation of the influence of male sex hormone on the alkaline and acid phosphatase activity of the seminal vesicles of the rat as determined by the method of Goetsch, Reynolds, and Bunting (1952) are presented in Tables 1 and 2 and Figures 5, 6, 7, and 8. A gradient of apparent alka-

TABLE 1. ALKALINE PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	0*	0	0	0	0	0
B. Cytoplasm	0	0	0	0	0	0
C. Secretion granules	0	0	0	0	0	0
Connective tissue (lamina propria)	+++	+++	++	++++	+++	+
Blood vessels in lamina propria	+++	+++	++	++++	+++	+
Smooth muscle	+	0	0	0	0	0
Blood vessels in smooth muscle	+	++	+	+++	+	+

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

line phosphatase reaction occurred adjacent to the interacinar stroma in sections deparaffinized prior to incubation which makes critical evaluation of the localization difficult. Furthermore, the acid phosphatase reaction in deparaffinized sections was somewhat variable. Incubation of non-deparaffinized tissue sections gave a more accurate localization of enzyme activity. No alkaline or acid phosphatase activity was found in air-dried smears of seminal vesicle secretion. These results confirm chemical

EXPLANATION OF PLATE I

FIG. 1. Basophilia in seminal vesicles of intact animal. Toluidine blue. ×160.

FIG. 2. Same as Fig. 1. Following ribonuclease prior to toluidine blue. ×160.

FIG. 3. Basophilia in seminal vesicles of intact animal. Eosin-methylene blue. ×500.

FIG. 4. Seminal vesicles of 20-day castrate. Atrophied secretory epithelium. Eosin-methylene blue. ×500.

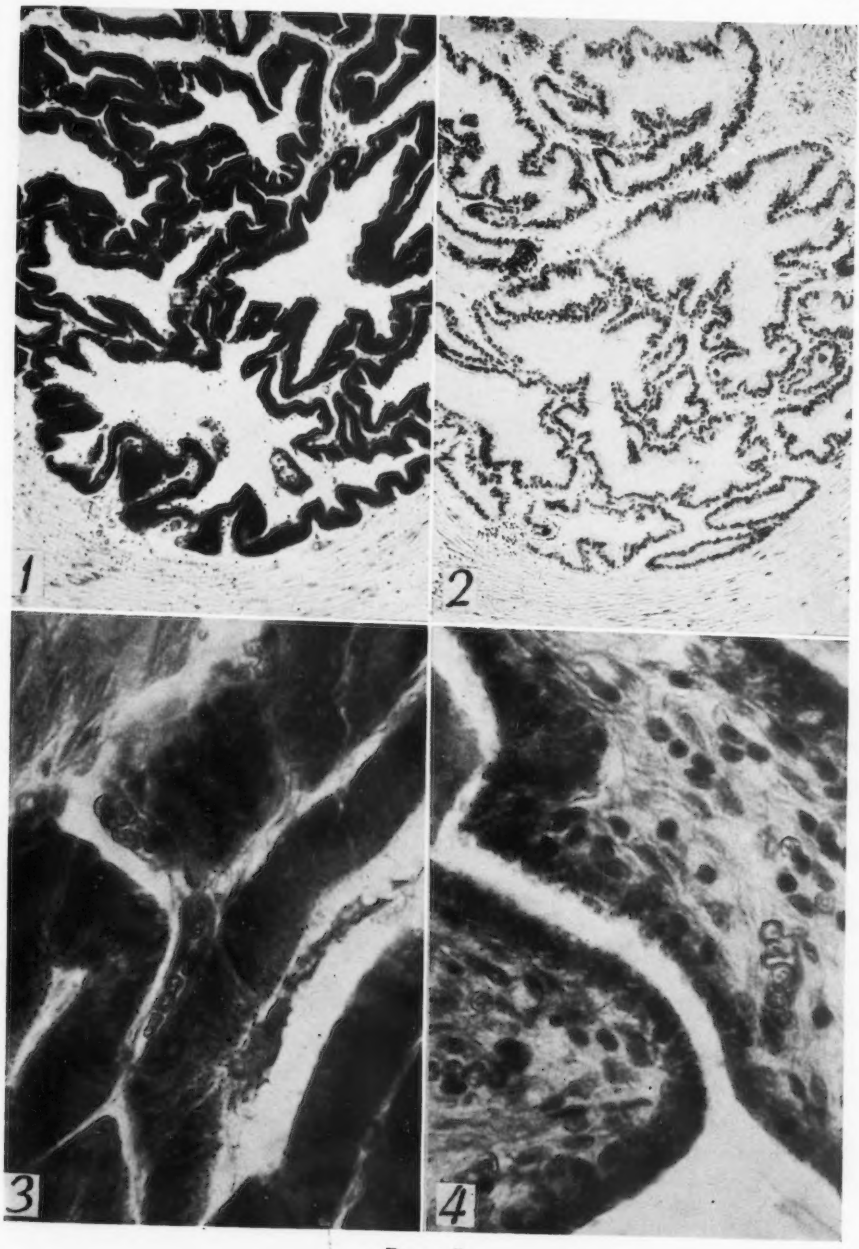

PLATE I

analyses which indicated the absence of both enzymes in the secretion of the rat.

Connective tissue. The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The lamina propria was formed chiefly of elastic fibers, and numerous arterioles and

TABLE 2. ACID PHOSPHATASE LOCALIZATION IN SEMINAL VESICLES OF INTACT, CASTRATE AND HORMONE-TREATED CASTRATE RATS

	Intact animal	10-day castrate	20-day castrate	5-day injected	10-day injected	20-day injected
Epithelium						
A. Nucleus	+++*	++	+	+++	+++	+++
B. Cytoplasm	+++	†	†	+++	+++	+++
C. Secretion granules	+++	0	0	++++	++++	++++
Connective tissue (lamina propria)	+++	++	+	++++	++++	++++
Blood vessels in lamina propria	+++	++	+ or ++	++++	++++	++++
Smooth muscle	++	+	+	+++	+++	+++
Blood vessels in smooth muscle	++	++	+ or ++	+++ or ++++	+++	+++

* Evaluation: ++++, intense; +++, strong; ++, weak; +, faint; 0, no reaction.

† Reaction appeared to decrease but because of the small amount of cytoplasm present in each cell it was difficult to evaluate the intensity of the reaction.

venules were widely distributed throughout this structure. No basement membrane was visible as such and the epithelium was seen to rest directly on the connective tissue as previously described by Moore, Hughes, and Gallagher (1930). The lamina propria divided into secondary and tertiary

EXPLANATION OF PLATE II

FIG. 5. Alkaline phosphatase localization in connective tissue of seminal vesicles of intact animal. No reaction in epithelium and secretion in lumen. Two hours' incubation. ×160.

FIG. 6. Alkaline phosphatase in the seminal vesicles of a 5-day T. P. injected castrate. A stronger reaction was obtained in the connective tissue of this stage than in Fig. 5. Two hours' incubation. ×160.

FIG. 7. Seminal vesicle tissue of a 20-day castrate showing acid phosphatase localization. Four hours' incubation. ×160.

FIG. 8. Acid phosphatase in the seminal vesicles of a 5-day T.P. treated castrate. Intense activity at the apical end of the epithelium. Four hours' incubation. ×160.

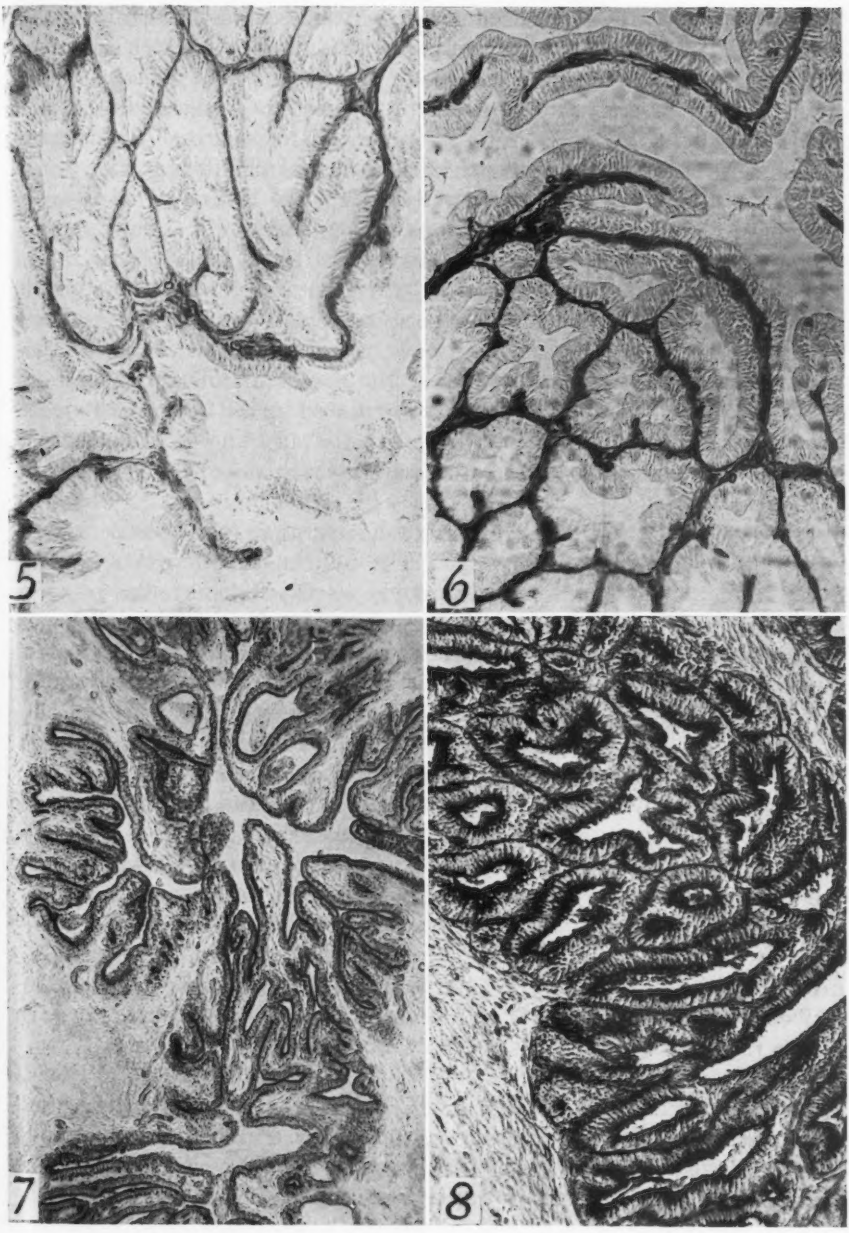

PLATE II

folds upon which the secretory epithelium was located. In these animals connective tissue appeared relatively thin and compact and this was probably due to the distension of the gland. Following Mallory's triple stain, both the nucleus and the cytoplasm of the secretory epithelium stained red. It was possible, however, to differentiate between these two cellular components in as much as the nuclei demonstrated a lighter reaction to the stain. The nuclei were located basally within the cell and the nucleoli, varying in number from 1 to 2, gave a strong, dark red reaction.

The general cytological picture obtained after 2 days of castration was similar to that of intact animals. However, there were a few visible minor differences. At this stage the secretory epithelium showed evidence of reduction in height when compared to that of intact rats. Visible changes were not observed in nuclei following the use of Mallory's triple stain. However, cytoplasmic alterations were observed in the form of a reduced amount of cytoplasm and almost complete disappearance of the secretory granules. Large amounts of secreted material were noted in the lumen of the glands.

Five days after castration it became evident that there was a marked reduction in the size of the epithelial cells and the columnar type present in the intact animal had been transformed into cuboidal. Secretion granules were not visible. However, some secreted material still remained within the lumen of the gland and the staining reaction of this varied from solid blue or a mixture of blue and red to one of completely red. The amount of connective tissue in the lamina propria and the smooth muscle surrounding it appeared to have increased, and it is suggested that this was the result of the atrophy of the epithelium as well as the decrease in the amount of secretion present which normally would have contributed to the distension of the gland. The cytoplasm of the secretory epithelium had atrophied considerably 10 days after castration. It was hardly possible to discern the cytoplasm as the epithelium had become cuboidal by this time. The muscle and connective tissue layers were thickened in appearance. In some cases there were a few residual patches of secretion left in the lumen. The same conditions existed in animals 20 days after castration as were described for the 10-day castrates. However, it appeared that there may have been a further reduction in the epithelial cytoplasm by this time.

In the case of the castrate which had been injected with T.P. for 2 days the epithelial cells had increased in height and secretion of varied tinctorial response was found within the lumen. Furthermore, secretory granules were absent from the cytoplasm. The gland as a whole had not increased extensively in size as was indicated in part by the relative thickening of the connective and smooth muscle layers. From this viewpoint, the glands remained quite similar to those of long term castrates. However,

the epithelial reactivation indicated that marked changes were taking place at this time. Examination of animals which had been treated for 5 days revealed what appeared to be indications of recovery from the changes induced by castration. Some secretion was visible in the lumen of the gland, but it was not possible to distinguish secretory granules as such in these preparations. The connective tissue appeared compact and the muscle layer was distended again and did not appear to occupy the bulk of the space as was observed in the shrunken glands of castrate animals. The seminal vesicles of the 10-day injected animals had the appearance of intact rats. Secretion was observed in the lumen of the gland, and secretory granules were visible in the apex of the stratified columnar epithelium. The connective tissue and the muscle layers were comparable to those of intact animals. The histology of the seminal vesicles of animals receiving the hormone for 20 days was similar to that of animals after a 10-day period of injection.

Periodic acid-reactive carbohydrates. By application of the periodic acid-Schiff technique to the seminal vesicles of intact animals, it was found that the epithelial nuclei and the secretory material within the lumen of the gland were unstained; however, a slight positive reaction was visible in the cytoplasm of some of the secretory epithelial cells. The smooth muscle layer and the elastica interna of the arterioles reacted weakly. A slight positive reaction was also observed in the fibers of the lamina propria. Glycogen was absent from seminal vesicle tissue of the rat. According to Leblond (1950) the seminal vesicles of the rat showed little reaction in the epithelium for periodic acid-reactive carbohydrates. Wislocki (1949) found glycogen in the seminal vesicles of deer. In specimens from the fall months it was present in the secretion, in the lumen of some of the acini as well as also in the cytoplasm of some of the epithelial cells. In June specimens none was found in the alveoli, but some was present in the cytoplasm of many of the epithelial cells.

Colchicinic mitoses. By use of the colchicine technique on intact animals, as well as 1-, 2-, and 20-day T.P. treated castrates, it was possible to determine the per cent of mitotically dividing cells in the secretory epithelium of seminal vesicle tissue. It was found in both intact animals and 20-day T.P. treated castrates that 0.3% of the cells showed mitoses. After one day of injection there was 0.2% dividing cells but following 2 days of hormone treatment there was 13.4%. This marked increase in mitotically dividing cells occurred at the time of the significant increase in QO_2 of seminal vesicle tissue from castrates receiving the hormone for the same period. It is of interest that the per cent (0.3%) of dividing cells was the same in the intact and 20-day castrate and the QO_2 was 2.7 in the former and 2.8 in the latter (Porter and Melampy, 1952).

DISCUSSION

In a biochemical investigation of the seminal vesicles of the rat (Porter and Melampy, 1952) it has been demonstrated that protein synthesis is an important function of this organ and is androgen controlled. The secretion produced by this organ in normal males contained 13.6% total nitrogen on a dry-weight basis. Castration of mature rats caused a decrease of 73% in the fresh weight of the secretion-free glands after 20 days and there was a 74% decrease in the total nitrogen content of this organ in the same interval. The catheptic activity decreased to 0.9 of a unit after 24 days in contrast to 2.8 units at the time of gonadectomy. Two days following castration there was a 78% decrease in the quantity of secretion initially present in the glands. The secretion had decreased 98% by the fifth day, 99% by the tenth day and was completely absent by the twentieth day. Subcutaneous injection of testosterone propionate (500 μg.) per day into castrate rats for 20 days resulted in the following increases per gland: wet weight 48 to 525 mg., nitrogen 0.2 to 7.3 mg. and the amount of secretion 0 to 1322 mg. The catheptic activity per gland of rats that were injected over a 10-day period increased from 0.9 to 4.5 units during this time.

In the present study it has been possible to extend these observations on the metabolism of the seminal vesicles by the application of histochemical methods for basophilia using toluidine blue and eosin-methylene blue. The presence of ribonucleoprotein in the secretory epithelium of this organ is suggested because the cytoplasmic and in many cases nucleolar basophilic reactions are abolished by treating sections with ribonuclease. This basophilia indicates an accumulation of ribonucleoprotein which may be related to the endocellular protein-synthesizing mechanism which is functional in this organ of the sexually mature rat. It is of interest that Wislocki (1949) has reported that cytoplasmic basophilia was absent from the epithelium of deer seminal vesicles but the cytoplasm had a strong affinity for acid dyes.

Recent investigations have shown a correlation between the nucleic acid content of cells of various tissues and their activity in protein synthesis. For example, in the pancreas, Caspersson (1950) has demonstrated that there is an intense protein metabolism and that the nucleotides of the nucleoli and cytoplasm are conspicuous. After pilocarpine stimulation there is a rapid synthesis of protein by the acinar cells which is preceded by nucleolar growth. Another example of this relationship is found in the gastric mucosa in which the protein-producing cells are characterized by a strong reaction for ribose nucleotides when compared to the parietal cells.

Deane (1946) in a study of the basophilia of hepatic cells of mice has demonstrated that the degree of the reaction is related to the metabolic

activities of the organ. For example, in the livers of mice killed after a known feeding time, changes in the amount and distribution of basophilic material occurred. Dempsey and Wislocki (1945) have shown that basophilia is a characteristic feature of the cytoplasm of the syncytium and cytotrophoblast of the human placenta. This reaction in the syncytium was most marked in early pregnancy and disappeared during the last months of gestation. Ribonucleoprotein was also reported as a component of the syncytial trophoblast of the cat and the trophoblast of the sow by Wislocki and Dempsey (1946 a, b). In an investigation of the histochemistry of the rodent's placenta Wislocki, Deane, and Dempsey (1946) observed cytoplasmic basophilia in the labyrinth and yolk sac of the mouse, rat, guinea pig, rabbit and hamster. It decreased gradually in the labyrinth but persisted unaltered in the yolk sac until term.

Rabinovitch, Junqueira, and Rothschild (1951) reported that testosterone propionate increased the amount of ribonucleic acid phosphorus of seminal vesicles of castrate rats. These investigators indicated that their findings support the suggestion of Caspersson (1950) dealing with the relation between ribonucleic acid and protein synthesis.

Lesher (1951) has observed that in the case of the salivary gland of Drosophila larvae there appears to be a close correlation between cytoplasmic basophilia and nucleolar size.

In the present study the nuclei in the secretory epithelium of the seminal vesicles of intact animals were large and elliptical and nucleoli were visible in most of them. Two days after gonadectomy a few of the nuclei were pyknotic and contained small nucleoli. Twenty days following castration many nuclei were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. However, in some instances, these structures were similar to those observed in intact animals and this variation may be due to differences in cellular activity. After 20 days of hormone injection, both nuclei and nucleoli of the secretory epithelium resembled those described as characteristic for intact animals. The changes observed in the nucleolus suggest that this structure is of importance in the secretory activity of the epithelium of the seminal vesicles.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of seminal vesicles of intact animals as well as castrates and hormone-treated castrates (Table 1 and Fig. 5). The connective tissue, particularly the lamina propria and its associated blood vessels, gave a strong reaction which became progressively weaker 10 days after castration. However, after 5 days of hormone injection, the reaction of this tissue became intense but after this time this reaction decreased as is shown in Table 1. This apparent decrease in the histo-

chemical reaction may be related in part to the great enlargement of the acini at this time and as a result the stroma became stretched and relatively thin.

Alkaline phosphatase concentration as measured by its activity per gm. dry weight of secretion-free tissue varied during the 20 days of hormone treatment; this activity was greatest by the fifth day when the average wet weight of the gland was 203 mg., and after this time there was a decrease in activity which was associated with the increase in the size of the gland which was 525 mg. after 20 days of injection (Porter and Melampy, 1952). Due to the greater relative increase in the various constituents of the gland, the absolute activity of this enzyme per unit of weight became less from the fifth to the twentieth day of injection. An intense alkaline phosphatase reaction was observed by Dempsey, Greep, and Deane (1949) in the stroma and capillaries immediately underneath the epithelium of the seminal vesicle of the rat. After hypophysectomy the phosphatase activity declined and finally disappeared, but the rate of disappearance varied with different substrates. Similar results followed gonadectomy. Wislocki (1949) observed in the case of October deer alkaline phosphatase at the distal margins of the epithelial cells of seminal vesicle tissue, in the secretion filling the lumen, and in the interacinar stroma. In June deer much less enzyme activity was present.

The absence of this enzyme from the secretory epithelium of the seminal vesicles of the rat is in accordance with the findings of other investigators which indicate the absence or low concentration of alkaline phosphatase activity in the cytoplasm of cells responsible for globular protein secretion such as in salivary glands (Gomori, 1941), pancreas (Kabat and Furth, 1941), gastric mucosa (Jacoby, 1946), and albumen-secreting regions of the fowl oviduct (Bradfield, 1949). However, the occurrence of this enzyme in the interacinar stroma of this gland is in agreement with the results of earlier studies on collagen formation in wounds (Fell and Danielli, 1943) and bones (Bourne, 1942, 1943), rat uterine epithelium (Pritchard, 1947), the vaginal wall of ovariectomized mice treated with estrogen (Jenner, 1947), and silk glands of spiders and caterpillars (Bradfield, 1950). According to Bradfield (1950) the presence of alkaline phosphatase in these various tissues suggests a relationship between this enzyme and fibrillar protein formation. However, in a recent study Bern and Levy (1952) have presented data which cast doubt on the concept of a relationship between alkaline phosphatase activity and the synthesis of fibrous proteins such as keratin, collagen, and myosin.

In the intact animal the nucleus of the epithelium showed a strong reaction for acid phosphatase which became progressively weaker during the 20 days following castration as shown in Table 2. The cytoplasm and secre-

tion granules likewise gave a strong reaction in the intact animal, but, as the cell atrophied following castration, the intensity of the reaction appeared to decrease in the cytoplasm and no secretion granules were present (Fig. 7). However, 5 days after hormone treatment the reaction was strong in the nucleus and cytoplasm and intense in the granules. The connective tissue and smooth muscle as well as the blood vessels in these tissues showed a decrease in acid phosphatase after castration followed by an increased reaction after hormone treatment (Table 2 and Fig. 8).

Porter and Melampy (1952) found that the acid phosphatase activity on a gland basis as determined by chemical analysis showed a 40% decrease 5 days following castration and a 65% reduction after 20 days. Likewise, during the same interval, alkaline phosphatase activity decreased 81%. There was a striking similarity between the decrease in activities of these enzymes and the loss in weight of the gland. The concentration of acid phosphatase increased 964% after 10 days of hormone injection.

Wislocki (1949) observed that acid phosphatase was localized almost entirely in the nuclei of epithelial cells and was found to a lesser degree in the nuclei of the stroma of the seminal vesicles of the deer. In the case of a June deer the acid phosphatase reaction was very much fainter than that found in October deer. Dounce (1950) has stated that acid phosphatase appeared to be generally present in both cytoplasm and nuclei. Wachstein (1944) in an investigation of renal phosphatases suggested that acid phosphatase was probably of importance in cell metabolism. This concept received further support when a marked increase in the enzyme was found in the perikaryon during chromatolysis (Bodian and Mellors, 1945), and Hard and Lassek (1946) have indicated that acid phosphatase may be of importance in maintaining the myelin sheath. The physiological significance of acid phosphatase in the tissues of the seminal vesicles of the rat is unknown but the activity of this enzyme was observed to be influenced either directly or indirectly by male-sex hormone.

SUMMARY

A basophilic reaction was observed in the cytoplasm of the secretory epithelium of the seminal vesicles of the rat following eosin-methylene blue and toluidine blue which was abolished by ribonuclease. The nucleoli were also basophilic but the reaction following ribonuclease was variable.

Alkaline phosphatase activity was absent from the nuclei, cytoplasm and secretory granules of the epithelium of the seminal vesicles of intact animals as well as castrate and hormone-treated castrates. The connective tissue, particularly the lamina propria and its blood vessels gave a strong reaction which became progressively weaker 10 days after castration.

Following 5 days hormone treatment the reaction became intense but after this time decreased. The results obtained from this histochemical study of both acid and alkaline phosphatase activity in seminal vesicle tissue agree with the chemical analysis of similar tissues for these enzymes. These enzymes were absent from seminal vesicle secretion. In the intact animal the nuclei of the secretory epithelium showed a strong reaction for acid phosphatase activity which became weaker following castration. Likewise the cytoplasm and secretion granules gave a strong reaction and the intensity appeared to decrease in the cytoplasm as the cells atrophied. However, after 5 days treatment with testosterone propionate (500 μg. per day) the reaction was strong in the nucleus and cytoplasm and intense in the granules. A similar response was also observed for acid phosphatase activity in the connective tissue, blood vessels, and smooth muscle of the gland.

The outer layer of the seminal vesicles of intact animals was composed of a fine, thin capsule of connective tissue; whereas the smooth muscle zone surrounding the lamina propria (interacinar stroma) was relatively thick and was interwoven with elastic tissue. The sites of localization of alkaline phosphatase closely paralleled the distribution of the lamina propria in this gland. Following castration there was an atrophy of the secretory epithelium and the connective tissue and muscle layers were thickened in appearance. The administration of testosterone propionate caused a reactivation of the gland.

Glycogen was absent from the seminal vesicle tissue of the rat. A slight positive reaction for periodic acid-reactive carbohydrates was obtained in the cytoplasm of some of the cells of the secretory epithelium, smooth muscle layer, the elastica interna of the blood vessels, and lamina propria.

It was found by the use of the colchicine technique in both intact animals and 20-day hormone treated castrates that 0.3% of the secretory cells showed mitoses. After one day of injection there was 0.2% dividing cells but following 2 days of hormone treatment there was 13.4%. Two days after gonadectomy a few of the nuclei of the secretory cells were pyknotic and contained small nucleoli; whereas 18 days later many were pyknotic and in numerous cases the nucleoli were absent or greatly reduced in size. After hormone treatment, both structures resembled those of intact animals.

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EFFECT OF IODIDE AND THYROID ON THE PRODUCTION OF TUMORS OF THE THYROID AND PITUITARY BY PROPYLTHIOURACIL[1]

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IN EXPERIMENTS carried out in this laboratory benign and metastasizing adenomatous tumors of the thyroid gland of rats have been found following prolonged administration (over one year) of propylthiouracil. Similar results have been reported previously by other investigators with thicurea (Purves and Griesbach, 1947; Money and Rawson, 1947), and with thiouracil (Dalton, Morris and Dubnik, 1948; Money and Rawson, 1950). The pathogenesis of the tumors is probably related to an increased production of thyrotropic hormone by the anterior pituitary over a prolonged period. The production or release of other hormones may also be affected, with resulting changes in other endocrine glands.

Ferguson and Sellers (1949) found that the concurrent administration of iodide with thiouracil for short periods (2–6 weeks) lessened the goitrogenic effect and resulted in thyroid glands which were 20–30% lighter than those produced by the goitrogen alone. They attributed this effect to the antithyrotropic action of iodide. This suggested that giving iodide with a goitrogen for a long period might affect the development of tumors produced by the goitrogen. Because of the antithyrotropic activity of the thyroid hormone itself, and in an attempt to maintain the metabolic and growth rates normal, in the experiments to be described dried thyroid was also given with propylthiouracil, the goitrogen selected for the investigation. The result of this treatment was an even greater enlargement of the thyroid gland, and in addition the occurrence of multiple adenomas of the anterior lobe of the pituitary.

EXPERIMENTAL

Two hundred and eighty rats of the Wistar strain, of equal sex distribution, bred in the laboratory, were divided into four groups and were given food and water *ad libitum*. Twenty from each group were kept in individual cages in order to follow the daily intake of food, and the remainder were kept in groups of ten in a cage. Each animal was weighed at weekly intervals.

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[2] Holder of a National Research Council of Canada Medical Fellowship, 1951–52.

Group 0 was fed the usual laboratory ration[3] in powdered form.

Group 1, laboratory ration plus propylthiouracil 0.02%.

Group 2, laboratory ration plus propylthiouracil 0.02% plus sodium iodide 0.02%.

Group 3, laboratory ration plus propylthiouracil 0.02% plus dried thyroid[4] 0.02%

The rats were maintained on this regime for 15 months after which time four rats from each group, chosen at random, were sacrificed at weekly intervals. At necropsy, body and organ weights were recorded and tissues were fixed in Bouin's solution or in formol-saline. After sectioning, tissues were stained with haematoxylin and eosin invariably, and in many cases with special stains for connective tissue, fat, ceroid, cytoplasmic granules, mast cells, etc. The pituitaries were stained with acid fuchsin, methyl blue (Wilson 1952) and often with Gomori's aldehyde-fuchsin stain. (Gomori, 1950). During the experiment the oxygen consumption of representative animals was measured at intervals using the method previously referred to by Sellers and You (1950). The level of iodine bound to plasma-protein was estimated (Barker, 1951) prior to sacrifice in representative groups, and the total iodine content of the thyroid (Kendall, 1920) was measured using that part of the gland not required for histological purposes.

Measurement of radio-iodine uptake (I^{131}) was carried out, and radioautographs were prepared following the method of Leblond and Gross (1948).

Measurement of exophthalmos

At necropsy, photographs were taken from vertically above the head of each rat after removal of the skin. The presence of exophthalmos was measured by dividing the distance between the points of maximum curvature on each cornea, by the distance between the malar processes. If the result was greater than 1, exophthalmos was said to be present; if less than 1, there was no exophthalmos. If the ratio was 1, or the angle of the head made measurements inaccurate, the reading was classed as "doubtful." The results are shown in table 4.

RESULTS

In analyzing the data it was found that expressing the results as a median and range presented more information in concise form than did the average and standard deviation. Occasional very large values rendered the latter method unwieldy, and difficult to interpret.

Rats which received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) gained very little weight during the entire experiment. The fur was coarse and sparse. The weight-curve of the group which received propylthiouracil plus thyroid extract (group 3) resembled that of the controls and at the end of the experiment the average weight was only slightly less than the control value (Table 1). The fur was normally sleek in appearance. In all groups the average food intake was consistent with the average gain in weight, the controls, and rats receiving propylthiouracil plus thyroid consuming almost twice the amount eaten by the other groups.

[3] Fox breeder cubes, Master Feeds, Toronto Elevators Limited, containing 2.6 mg. iodine per 100 gm.

[4] Dried thyroid powder containing 0.3% iodine, Parke-Davis and Company, Walkerville, Ontario.

Thyroid

Weights: (Table 1). The addition of propylthiouracil to the diet caused a significant increase in the weight of the thyroid gland. When propylthiouracil and iodide were administered simultaneously the increase in weight was less than that observed with propylthiouracil alone, but when propylthiouracil and thyroid extract were administered simultaneously (group 3), the thyroid glands were tremendously enlarged to as much as eighty, and on the average to twenty times the weight of the thyroids of the control animals.

Pathology. Gross: The thyroid glands from animals of the three groups which had been fed propylthiouracil were all enlarged to a variable ex-

TABLE 1. WEIGHTS

No. of rats	Group	Sex	Body weight in grams			Thyroid weight, mg.		Pituitary weight, mg.		Adrenal weight, mg.	
			Initial av.	Final							
				Median	Range	Median	Range	Median	Range	Median	Range
15	0	M	152	399	340–484	36.0	27.4– 47.4	11.8	9.2– 18.0	37.9	27.2–45.8
14	control	F	144	275	254–324	28.5	20.2– 39.0	14.7	10.8– 35.7	55.6	28.0–78.8
20	1	M	159	191	142–278	97.7	44.6– 395.2	12.2	7.8– 17.0	24.0	18.1–32.1
17	PTU	F	145	175	119–230	79.8	40.2– 568.0	15.0	9.0– 22.4	24.2	18.2–36.6
14	2	M	166	189	148–211	50.1	27.6– 143.4	11.6	8.2– 15.6	24.2	19.2–29.0
12	PTU+I	F	152	158	108–212	48.6	32.0– 94.0	15.3	7.8–500.0	24.8	18.0–34.6
12	3	M	152	346	278–402	517.1	363.8– 674.1	20.9	10.0–164.0	39.9	32.4–54.0
12	PTU+Th	F	144	257	154–302	442.2	150.0–1660.0	28.6	11.6– 60.6	68.8	42.0–89.4

tent. In some cases the enlargement was diffuse, regular and involved both lobes. In others, one or both lobes had multiple small firm nodules or an occasional marble-sized cystic swelling (Fig. 1). The color was also variable, although glands from groups 1 and 3 (propylthiouracil and propylthiouracil plus thyroid) were deeper red and apparently more vascular than glands from group 2 (propylthiouracil plus iodide).

Microscopic: The microscopic appearance varied greatly within and between treated groups. Large areas (comprising most of the gland) showing

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EXPLANATION OF PLATE I

FIG. 1. Thyroid (wt. 1660 mg.) and pituitary (wt. 22.8 mg.) glands. Propylthiouracil plus thyroid group. Thyroid metastases found in lung. Note small nodule on pituitary (upper right, see figs. 11, 13).

FIG. 2. Pituitary gland (wt. 38 mg.) same group. Note nodule attached to anterior lobe (entire left of photograph). See figs. 16, 17.

FIG. 3. Thyroid. Small circumscribed area containing colloid. (Mallory×40.)

FIG. 4. Thyroid. Colloid "lake." Compressed follicular cells form a pseudo-capsule. (H.+E.×40.)

FIG. 5. Thyroid. Follicular cells surrounding well-stained colloid are stratified and hyperchromatic. (H.+E.×560.)

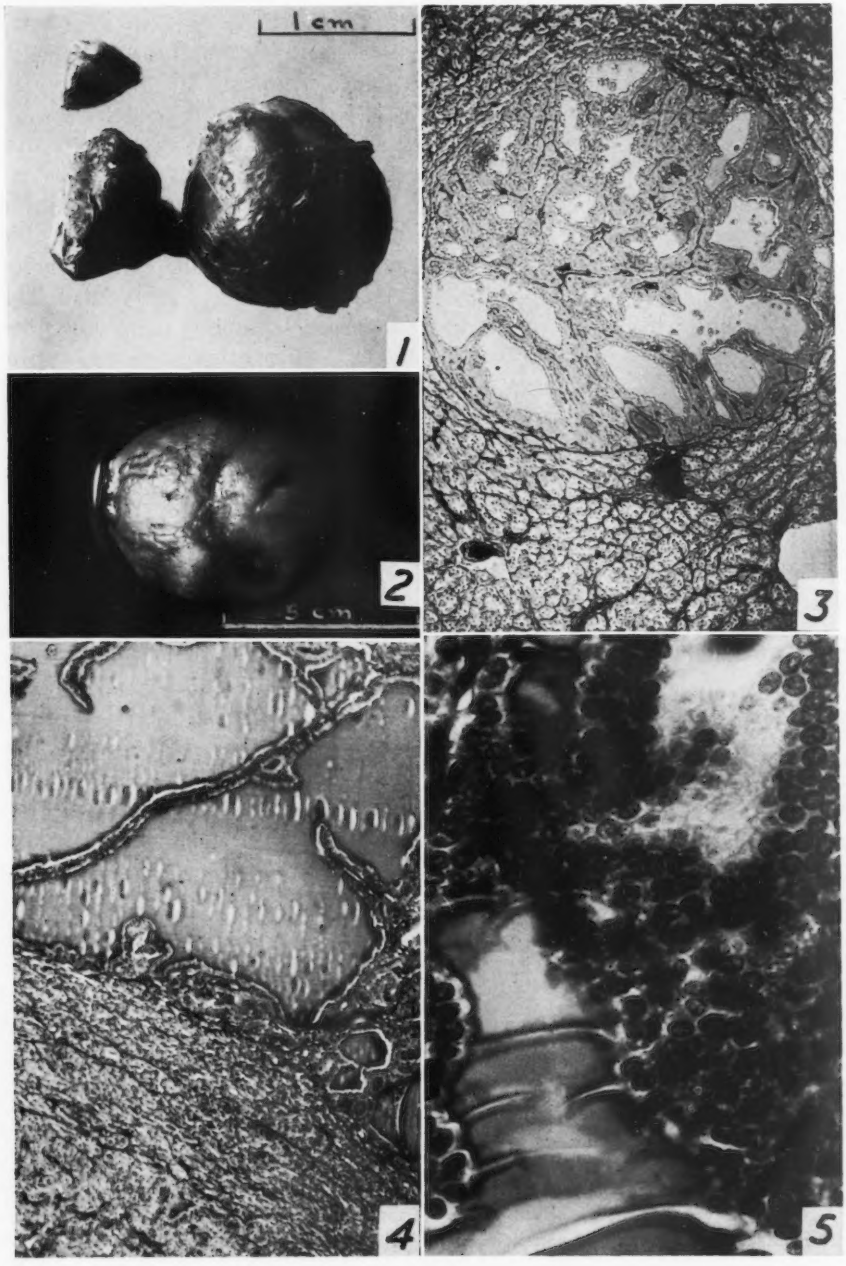

PLATE I

hypertrophy and hyperplasia of the follicular cells, mitotic figures, budding of the follicular wall (with an apparent increase in number of follicles), vacuolation of, or more frequently almost complete disappearance of colloid could be found in every section. Generally these changes were advanced so that the normal architecture of the gland was no longer discernible.

Frequently (in almost every gland weighing more than double the average weight of the normal) an area made up of large follicles containing well stained colloid was demarcated from its surroundings by a pseudocapsule made up of compressed follicular cells or of fibrous trabeculae. The cells within such a nodule showed considerable variation in that sometimes they appeared cuboidal or even flattened, and in other sections, hyperchromatic, columnar cells in layers with many mitotic figures projected and branched into large colloid cysts or "lakes." Wide blood-sinuses, and evidence of hemorrhage into the cysts could be seen. The similarity of some areas to the papillary cystadenomas of human thyroids was a striking feature. Less frequently, circumscribed or semi-circumscribed areas, with little or no colloid and either vacuolated, but hyperchromatic cells, or anaplastic cells were evident (Figs. 3–7).

While the size of the glands (and of gross colloid cysts) was much greater in the group receiving propylthiouracil with thyroid the *presence* of localized colloid areas, of colloid cysts, or of cellular adenomas was not restricted to any one group receiving propylthiouracil. By histological examination it was not possible to assign any tissue-section chosen at random to its correct group.

In 3 or perhaps 4 animals (one—group 1, propylthiouracil; 2 or 3—group 3, propylthiouracil plus thyroid) metastases were found seeded through the lungs. Thyroid follicles containing colloid were found invading the capsule of the gland, in venules in muscle adjacent to the gland, in the

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EXPLANATION OF PLATE II

FIG. 6. Thyroid. Projections from the follicular walls extend into dilated blood sinuses. Note the thin "stem" of the daughter follicle (center). (H.+E. ×150.)

FIG. 7. Thyroid. Hemorrhage has occurred into follicles. In some follicles (upper) necrosed follicular cells, blood pigments and colloid are present. Organization of smaller follicles has taken place. (H.+E. ×40.)

FIG. 8. A follicle of thyroid tissue in a venule adjacent to the gland. Note the intimal layer of the vessel, the red blood cells between it and the follicle, the colloid in the follicle. (Mallory ×900.)

FIG. 9. Thyroid tissue in the submucosa of the esophagus. The epithelium (lower left) and muscular layer (upper) can be seen on either side of the thyroid tissue. (Mallory ×150.)

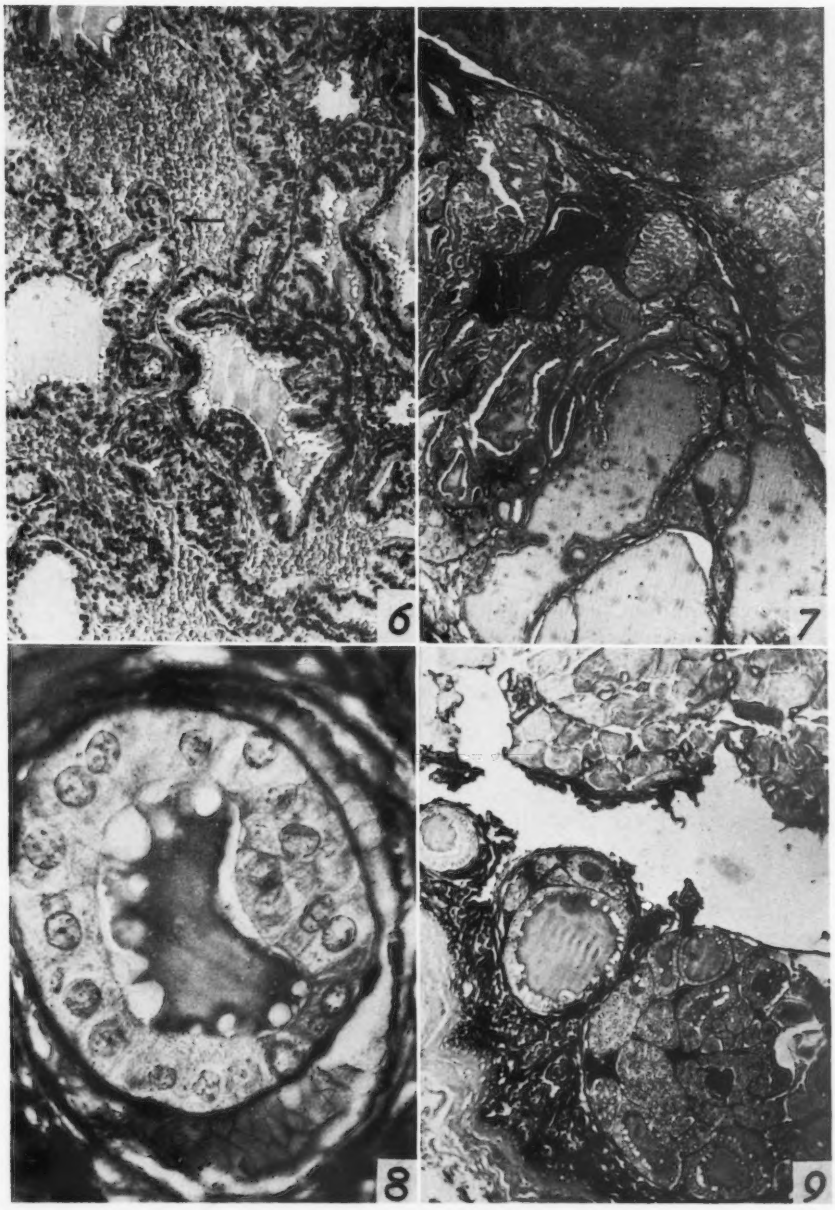

PLATE II

submucosa of the esophagus and in muscle deep to the esophagus, in a branch of the pulmonary artery in the lung and in numerous alveolar vessels (Figs. 8–10). In some instances the metastasizing thyroid tissue resembled normal gland while in other hyperchromatism, stratification, and frequent mitotic figures were seen.

Physiological and Biochemical Studies. Metabolic rates and protein-bound iodine: (Table 2)

The addition of propylthiouracil to the diet resulted in a fall in the metabolic rate and plasma protein-bound iodine values to hypothyroid levels. In group 2 (propylthiouracil plus iodide) the metabolic rate was

TABLE 2. METABOLIC RATE AND PLASMA PROTEIN-BOUND IODINE VALUES

Group	No. of rats	Metabolic rate		No. of rats	Protein-bound iodine	
		Average MR* (15 months)	% decreased from normal		Median, μg.	S.D.
0	16	150	0	13	4.63	±1.17
1	8	99	34	15	1.93	±0.61
2	7	65	57	12	6.93	±1.53
3	8	138	8	10	2.55	±0.62

* Cc. O_2/min./m^2 at N.T.P.

even lower, but the protein-bound iodine was above the control value. The values obtained for rats which had received propylthiouracil plus dried thyroid (group 3) were slightly below those of the controls.

Radio-active iodine uptake:

The uptake of radio-active iodine (24 hours) calculated on a basis of concentration per unit of thyroid tissue was diminished in the rats which had been fed propylthiouracil (group 1). The uptake was less in the rats which had been fed propylthiouracil plus thyroid extract (group 3) and least in those rats which had received propylthiouracil plus iodide (group 2).

EXPLANATION OF PLATE III

FIG. 10. Lung. Thyroid tissue in a branch of the pulmonary artery. The muscular wall of the vessel is seen in the lower left, and bronchial epithelium adjacent to the artery, in the upper right. (Mallory×150.)

FIG. 11. Pituitary (see Fig. 1). Localized areas of chromophobic cells found in many rats of the propylthiouracil-thyroid group. (Acid fuchsin, methyl blue×40.)

FIG. 12. Pituitary. Edge of a chromophobic area (right). The cytoplasm of the pseudocapsular cells is compressed and stains indefinitely, the nuclei are pyknotic. (Acid fuchsin, methyl blue×500.)

FIG. 13. Pituitary. A higher magnification of chromophobic area shown in Fig. 11. Both cytoplasm and nuclei stain poorly, vary in size and shape. In the photograph red blood cells appear black, one basophil can be seen (upper right). (Acid fuchsin, methyl blue×500.)

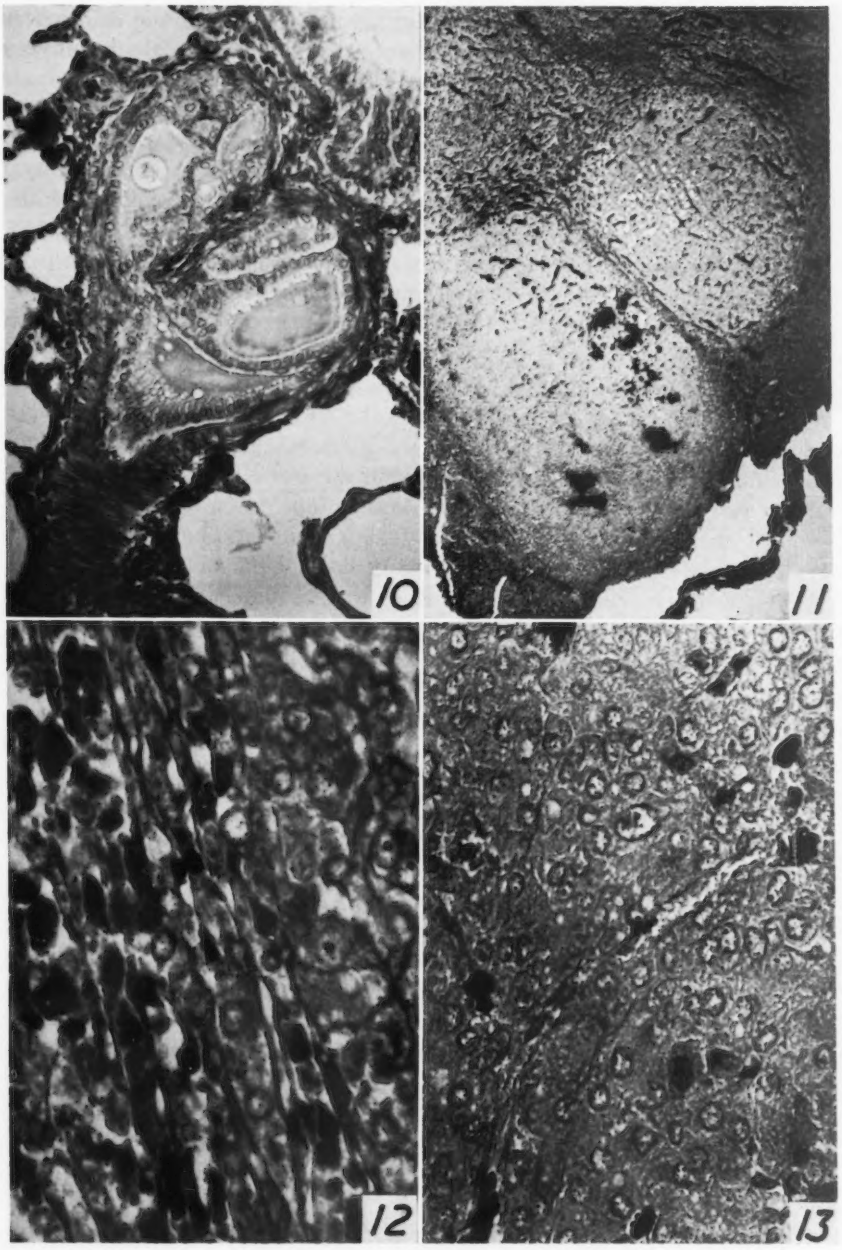

PLATE III

Although only six animals in each group were studied, the differences were significant. No significant difference existed when the calculation was carried out for total weight of thyroid tissue.

Radioautographs (24 hours) showed little or no concentration in the three groups which received propylthiouracil. The difference between groups, if any, could not be quantitated satisfactorily. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas.

Iodine content of the thyroid gland: (Table 3)

The iodine content of the thyroid gland was very low in the rats which had been fed propylthiouracil (group 1). The level of iodine was higher in

TABLE 3. IODINE CONTENT OF THYROID

Group	No. of rats	Iodine content (μg. per 100 mg. wet tissue)		Iodine content (μg. iodine per gland)	
		Median	Range	Median	Range
0	21	130.4	88.4 –196.40	46.6	18.2 –58. 3
1	29	2.41	0.41– 11.40	2.74	1.08– 6.86
2	18	23.68	8.32– 39.89	11.83	7.20–20.40
3	13	5.64	2.60– 10.19	27.68	21.50–43.20

those rats that had received propylthiouracil plus iodide (group 2). In the group which had been given propylthiouracil plus thyroid extract the iodine content of the whole thyroid was found to be only one-third less than that of the controls, although the value was very low when calculated per unit weight of thyroid tissue.

Pituitary

Weights: (Table 1). The pituitaries of the rats which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) were no heavier than those of the control animals. The addition of thyroid extract to the propylthiouracil (group 3) caused a significant increase in the weight of the pituitaries.

Pathology. Two of the pituitary glands from rats which received propylthiouracil plus thyroid extract (and one, the largest of all, from a rat receiving propylthiouracil plus iodide) were large (pea-sized), friable and dark red in color. Most of the glands from this group (Figs. 1 and 2), although somewhat larger than usual, looked normal except that in many instances there were one or more small flattened reddish nodules on the infero-lateral surfaces of the anterior lobe. The larger of these were as big as a normal pituitary (rat).

Microscopic: There was a consistent degranulation of the eosinophil cells in the treated groups which varied in degree from animal to animal. In spite of this it was usually, but not invariably, possible to find some

cells which contained eosinophilic granules in part of a section. Basophils were almost always degranulated; and often the cells were enlarged and the cytoplasm was vacuolated. In most sections from animals of treated groups a considerable number of very large cells showing extreme vacuolation of the cytoplasm but no granulation could be seen. The staining characteristics of the cytoplasm varied but usually stained pale blue with basic stains.

There was a relative increase in chromophobic cells.

In a high proportion of the propylthiouracil plus thyroid group, areas which stained very poorly were demarcated from normal tissue by a pseudocapsule of compressed cells (Figs. 11, 12). These areas were made up of masses of "chromophobic" cells, the nuclei of which varied in size and appearance from small, deeply stained, almost pyknotic, to very large hypochromatic structures. In a few sections faint eosinophilic granulation could be made out in the cytoplasm. The cytoplasm was pale, profuse and the cell borders were irregular (Figs. 13, 16, 17). The cytogenesis of the cells is not clear, and in spite of the chromophobic nature of the cells we are inclined to believe that they may have originated from chromophil cells. The evidence for this is not entirely convincing and consists of finding eosinophilic and less often basophilic transitional forms (Figs. 16, 17).

In the large hemorrhagic tumors found grossly, the microscopic appearance showed chromophobic cells interspersed with numerous mast cells (Figs. 14, 15). No "transitional" forms were seen in these instances. Areas of hemorrhage and necrosis were present and these contained deposits of "ceroid" demonstrable by special staining.

Adrenals

Weights: (Table 1). The adrenals of the female rats (but not the male) which had received propylthiouracil (group 1) or propylthiouracil plus iodide (group 2) weighed less than those of the control animals. Those rats which had received propylthiouracil plus thyroid extract had adrenal glands which weighed approximately the same as those of the control animals.

Pathology. The adrenal glands of most of the rats in the groups treated with propylthiouracil, or propylthiouracil plus iodide showed involutionary changes in the zona reticularis (Fig. 18). The addition of dried thyroid to the diet (group 3) prevented this involutionary change.

Exophthalmos

In Table 4 it is seen that a high percentage of animals receiving propylthiouracil became exophthalmic. Dried thyroid reduced the incidence very greatly but iodide was ineffective.

DISCUSSION

Rats which received propylthiouracil plus thyroid extract achieved an almost normal growth curve. This suggests that the depression of growth observed in the rats to which propylthiouracil or propylthiouracil plus iodide was given was due to a lack of thyroid hormone (Hammett, 1926) rather than to a direct inhibitory effect of proplythiouracil on the growth hormone of the anterior pituitary (Williams *et al.*, 1944).

The addition of a relatively large amount of iodide (approximately 1.5 mg. sodium iodide per rat per day) to the diet containing propylthiouracil reduced the goitrogenic effect of propylthiouracil, although the reduction of metabolic rate was even greater than in the group which received propylthiouracil alone.

TABLE 4. EXOPHTHALMOS

Group	No.	Exophthalmos			Incidence (per cent)
		Present	Absent	Doubtful	
0 (control)	25	0	24	1	0
1 (PTU)	35	29	4	2	83
2 (PTU+I)	22	17	4	1	77
3 (PTU+Th)	16	3	12	1	19

Thyroid

An unexpected finding was the tremendous enlargement of the thyroid gland when dried thyroid (approximately 3 mg. per day) was added to the diet containing propylthiouracil. This amount of thyroid had been chosen in an attempt to maintain the growth rate and metabolic rate within nor-

EXPLANATION OF PLATE IV

FIG. 14. Pituitary, Chromophobic adenoma. Variation in nuclear and cell size is apparent. Nuclear disintegration, extreme vacuolation of the cytoplasm and in other sections hemorrhage and necrosis are seen. The cells which appear black in photograph are mast cells. (Acid fuchsin, methyl blue×500.)

FIG. 15. Pituitary. Chromophobic adenoma. Large purple granules (black in photograph) cover the nuclei of cells having all the staining characteristics and appearance of mast cells. (Basic fuchsin—fast green×1000.)

FIG. 16. Pituitary (gross fig. 2). Chromophobic area with large vacuolated cells, showing no distinctive granulation, stained pale blue with basic stains. (Acid fuchsin, methyl blue×1280.)

FIG. 17. Another field of same pituitary showing transitional chromophil cells. Upper cells (B) are basophils; lower cells (A) are eosinophilic. (Acid fuchsin, methyl blue×1280.)

FIG. 18. Adrenal. At lower right the large pale chromaffin cells are normal medullary tissue. Approximating this area atrophied cells of the reticularis, surrounded by black reticular fibres can be seen. (Mallory×500.)

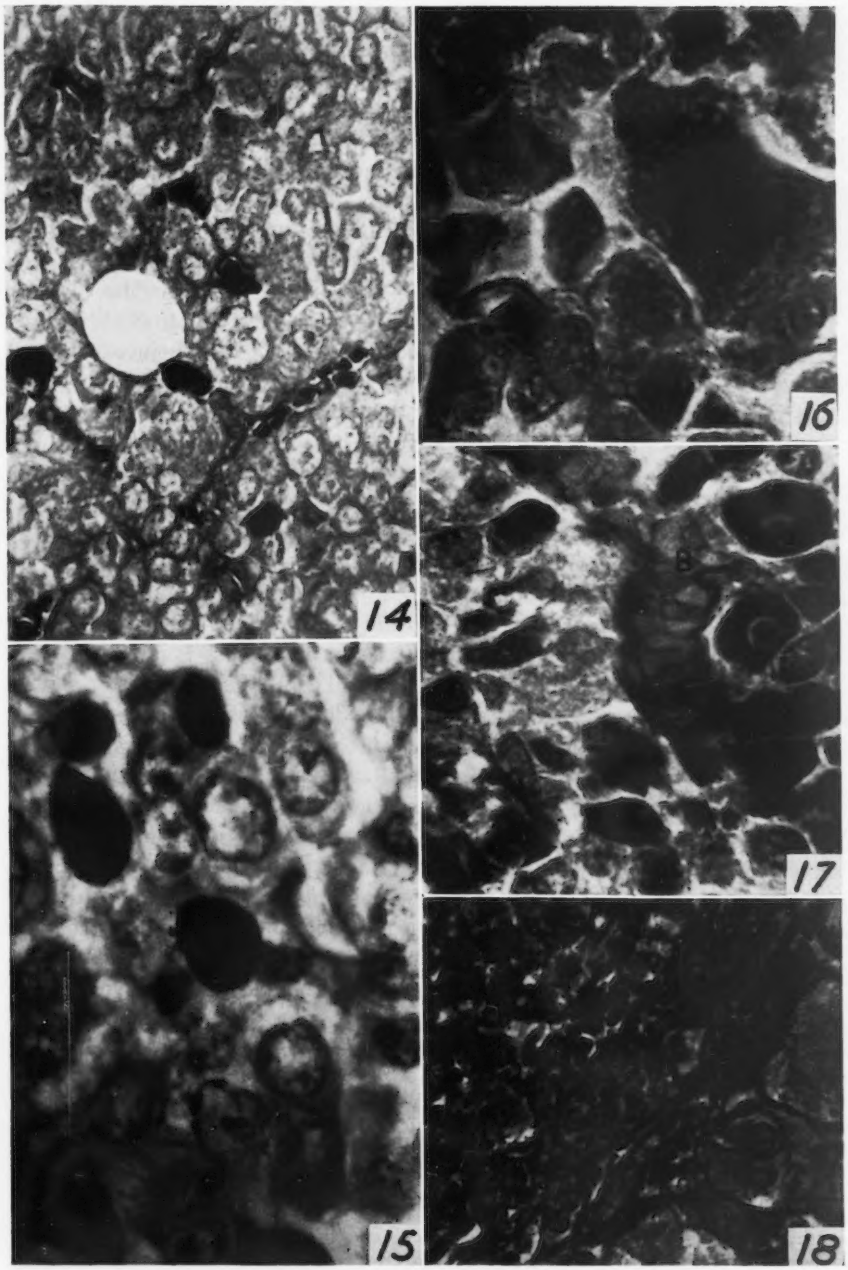

PLATE IV

mal limits in spite of the continued administration of propylthiouracil. Both of these values were slightly lower than those of the control group but fell within the range of "normal." Nevertheless, the size of the thyroid, and of the pituitary was significantly greater than in the other groups. It is necessary to postulate that there was some substance in the thyroid extract which stimulated the pituitary and the thyroid gland, or that the thyroid hormone supplied as dried thyroid in the diet was less than necessary to inhibit the pituitary, and a "hormonal imbalance" existed. Other evidence at hand, showing that in lengthy experiments of this nature, relatively large amounts of crystalline l-thyroxine added to the diet are necessary to prevent enlargement of the thyroid, suggests that the second alternative is more likely. It is possible that the thyroid hormone exerts a stimulatory function (under some circumstances) on the cells of the pituitary and thyroid gland. Unfortunately we have no information on the thyrotropin content of the anterior pituitary under the conditions described here. The great enlargement of the thyroid, and the production of metastasizing adenomas, suggest that continued stimulation of the thyroid gland by the thyrotropic hormone is the important causal factor (Purves and Griesbach, 1949; Money and Rawson, 1950).

The spread of the tumors appears to be through vascular channels. Apart from local invasion of the capsule of the gland, metastases were found exclusively as intact thyroid follicles in blood vessels in the thyroid, in venules adjacent to the thyroid, in branches of the pulmonary artery, and in alveolar vessels. No attempt was made to transplant or culture the tumors, but from other somewhat similar studies (Morris, Dalton and Green, 1951; Hall and Bielschowsky, 1949; Bielschowsky *et al.*, 1949) it would appear that they are at least *potentially* malignant.

Pituitary

The development of chromophobe adenomas of the pituitary in mice following destruction of the thyroid by radio-iodine has been reported by Furth (1952). As far as we know, no investigators have observed such tumors subsequent to the administration of goitrogenic drugs. Beyond compression of the base of the brain, not even the largest showed evidence of infiltration of adjacent tissue, or metastatic spread. The tumors were made up of chromophobic cells of very variable size interspersed with mast cells. Areas of hemorrhage and necrotic tissue (containing ceroid) were present in the larger ones. It has already been observed that in our opinion this staining characteristic does not necessarily mean that they arose from chromophobe cells. Indeed our present view tends to oppose this.

The metabolic rate and protein-bound iodine were lower in the group receiving propylthiouracil than in the controls. The lower metabolic rate

obtained when iodide was given in addition to propylthiouracil is difficult to explain but is perhaps related to the relatively high dose level of iodide. The higher value obtained for the protein-bound iodine in this group probably reflects the higher intake of iodide and has been observed by others (Barker, 1948). In the group receiving thyroid the protein-bound iodine value was lower than normal, which supports the hypothesis that the amount of thyroid given was inadequate to replace that normally supplied by the gland.

Iodine content of the thyroid

The low iodine content of the thyroids from animals which received propylthiouracil, and the absence of concentration of iodine shown by radioautography was not unexpected. The differences between groups (in iodine content) are appreciable but cannot be explained satisfactorily from available information. The finding of granular eosinophil cells in the pituitaries of *all* the treated groups would imply (Purves and Griesbach, 1947) that thyroidal or extrathyroidal production of some thyroid hormone was taking place in both the propylthiouracil and propylthiouracil plus iodide groups. If such were the case, differences in iodine take-up and content might be expected with the differing treatments.

Exophthalmos

The production of exopthalmos in rats by feeding propylthiouracil has been reported previously (Sellers and Ferguson, 1949). This finding which occurred in a high proportion of the animals treated with propylthiouracil, or propylthiouracil plus iodide, was much less common in those animals which were given dried thyroid. Without knowing the amounts of thyrotropin produced or released it is not possible to comment on its possible role in the causation of exophthalmos. From the size of the thyroid glands, one would assume that the amount of thyrotropin available was large. Nevertheless, administered thyroid effectively prevented the exophthalmos.

SUMMARY

For a period of 15 months or more Wistar rats were fed the usual laboratory ration supplemented with (1) propylthiouracil, 0.02%, (2) propylthiouracil plus 0.02% sodium iodide, (3) propylthiouracil plus 0.02% dried thyroid.

Growth and metabolic rates were much lower than normal with propylthiouracil or propylthiouracil plus iodide but were nearly normal with propylthiouracil plus thyroid.

The thyroid glands were enlarged greatly in the three treated groups; the differences in gland size between each group (including the control)

were significant. The thyroids of rats which received iodide showed the least enlargement and those of rats which received dried thyroid with propylthiouracil the greatest (up to 80 times normal).

Histologically, in addition to the hyperplasia and disappearance of colloid typical of propylthiouracil administration, circumscribed areas of large follicles containing colloid were present. The cellular detail and apparent "activity" in these adenomas varied greatly. The particular treatment did not appear to control the type of adenoma produced. The radioiodine uptake was low in treated groups. There was no apparent concentration of iodine in the deeply stained colloid of the adenomas (24 hour radioautographs).

In 3 or perhaps 4 cases metastatic thyroid tissue was found in the lungs. Spread was by vascular channels as metastases were found in venules of thyroid, esophagus, muscle, and in a branch of the pulmonary artery. No attempt at transplantation or culture was made.

The pituitaries of rats receiving propylthiouracil plus thyroid were significantly heavier than those of other groups. Single or multiple "chromophobic" adenomas occurred frequently in this group.

The eosinophils of the pituitaries of all treated groups were incompletely degranulated. Generally the basophils were degranulated, large and vacuolated.

It is suggested that the tremendous increase in size of the thyroid gland, the great enlargement and adenomas of the pituitary, in the propylthiouracil plus thyroid group may be ascribed to a hormonal imbalance rather than to some unknown substance in the dried thyroid. The thyroid hormone itself may exert some stimulating function on the cells of the pituitary and thyroid.

Exophthalmos occurred in most (about 80%) of the rats receiving propylthiouracil or propylthiouracil plus iodide. It occurred in about 20% of those receiving propylthiouracil plus thyroid.

The adrenal glands of female rats receiving propylthiouracil, propylthiouracil plus iodide, but not propylthiouracil plus thyroid, were significantly lighter than those of the controls. Histologically the reticularis showed involutionary changes.

Acknowledgment

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BIOASSAY OF RELAXIN

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IN 1926 Hisaw first demonstrated experimentally the relaxation of the pelvic ligaments of guinea pigs during estrus following the injection of blood serum from pregnant rabbits. Later work indicated a specific hormone, designated as relaxin, was responsible for the looseness of the symphysis pubis, since the effect was also observed following injection of extracts of sow corpora lutea into castrated guinea pigs provided the animals were pretreated with estrogen. Since that time, most of the experimental work on relaxin has been based upon bioassays with this all-or-none pelvic looseness endpoint determined by manual palpation. During this assay procedure, the possibility of traumatizing the loosened tissue of the pubic symphysis or exaggerating the hormone-induced separation cannot be excluded. The radiographic measurement of the inter-pubic gap would seem desirable because not only would this provide an objective record but the determination of the actual interpubic distance might provide a more sensitive assay. Hall (1947) and Talmage and Hurst (1950) have employed dental x-ray machines to demonstrate relaxin activity but few details of the actual technics employed have been published. Talmage and Hurst attempted to refine the conventional guinea pig assay by substituting radiographic measurements for manual manipulation. However, the variability of response of individual animals was so great that these investigators concluded the assay would be impractical. The use of x-rays to determine separation of the pubic symphysis seems justified because Hall (1947) has shown that in untreated pregnant mice the time of beginning of pubic separation coincides within a day or two with the first histological evidence of relaxin effects on the pubic tissues.

This paper represents an attempt to develop a more reliable bioassay for relaxin employing mice with radiographic evaluation of the response.

EXPERIMENTAL

Swiss albino virgin mice[1] were employed after ovariectomy, except in later trials when intact immature females were employed. After a five-

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[1] Albino Farms.

day postoperative period, each animal received daily intraperitoneal injections of 1.0 μg. estradiol benzoate in corn oil, usually for a total of eight days. This procedure was varied in some experiments as will be indicated. During the experiments where relaxin was injected over a period of more than one day, the estrogen injections were continued on a daily basis.

In order to standardize the positioning of the animal for radiography as well as to remove the operator from the area of harmful radiation, the holder, illustrated in Figure 1, was constructed.[2] In use, the lower abdomen of the mouse rests directly upon the film. The cephalic edge of the film is tilted upwards into the belly of the animal to approximate a right angle with the x-ray beam. The film left projecting from the rear of the holder conveniently is used for placing the lead numerals to identify the individual negatives. For the accurate radiographing of the inter-pubic gap, the x-ray beam must strike the symphysis sagitally and, in order to avoid the vertebral and tail bones, from some angle below the vertical. After the animal's hindquarters are fixed in the holder with the hinged restrainer bar, the tail is pulled upward and forward and wedged into the forked holder. This motion tilts the entire pelvis and tends to closely approximate the symphysis against the film. The focal spot of the x-ray tube was at least 35 cm. from the animal so that the image on the film could be assumed to represent the actual bony separation. Measurements obtained under these conditions agree closely with figures published by Hall (1948).

It was found that the consistent production of sharp radiographs which permitted accurate measurements was difficult. The x-rays pass through approximately 10–12 cm. of soft tissue and careful exposures were necessary to produce sufficient contrast with the thin bony parts for accurate visualization of the pubic gap. Since the sole aim of the x-ray technic was the production of an objective record of the separation of the pubic bones, the use of high kilovoltages, customarily employed in routine medical radiographic work to bring out detail, resulted in films with such gradual density transitions across the pubic symphysis that the precise placing of the caliper points was impossible. The quality of radiographic image required was the short-scale, high contrast type. The x-ray machine available for this work was a General Electric Portable, Model F, with a 10 milliampere capacity and a constant output of 63 kilovolts. By interposing a

[2] The holder was made from 0.250″ sheet of Plexiglass with the hinged restrainer bar bent to shape by the cautious application of heat. An ordinary snap fastener of the type usually employed on canvas awnings was used to keep this bar fastened down when in use. The film slot was built up of wedge-shaped pieces to position the dental films approximately 20° from the horizontal. Two thin rabbeted pieces were cemented to the edges of the inclined wedge to accommodate dental films inserted with the long diameter parallel wth the holder.

variable voltage transformer[3] between the power supply and the x-ray machine the primary voltages in the x-ray tube transformer could be varied and lower kilovoltages thereby obtained.

Numerous trials using various combinations of kilovoltages, exposure times and milliamperes indicated that an output of 33 kilovolts was required to produce a high contrast negative which allowed precise visualization of the articulation of the symphysis pubis. In order to produce adequate image density an exposure of from 2.5 to 3 seconds was necessary, depending on the size of the individual animals. The use of a more powerful machine (i.e., higher milliamperage) would of course, allow shorter ex-

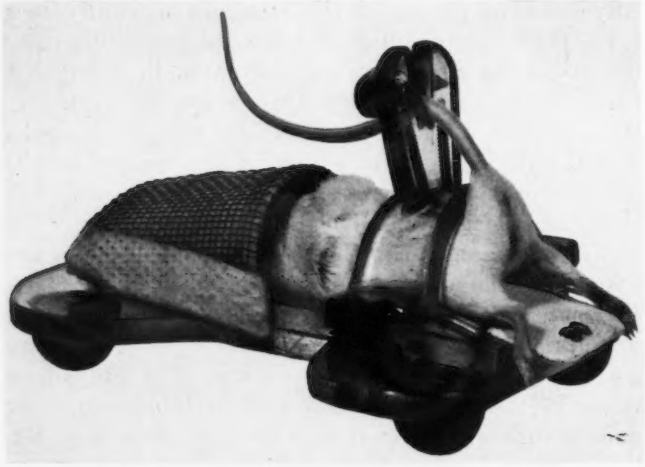

FIG. 1.

posure times since the product of milliamperes times seconds is a constant. Most of the available dental x-ray film emulsions were tried with apparently best results from Eastman Kodak Periapical Ultra-Speed emulsion. All measurements of the interpubic distance were made by the same individual (J.D.) using an inside micrometer caliper[4] reading in millimeters with the outside of the jaws precision ground to a conical point, the inner surfaces remaining flat. Each measurement represented the shortest distance between the pubic bones.

Before placing in the holder, all animals were lightly anesthetized with ether. The holder was carefully maintained in the same position relative to the x-ray machine and the hindlegs and pelvis of the animals spontaneously

[3] Powerstat, The Superior Electric Co., Bristol, Connecticut.

[4] Brown and Sharpe, Providence, R.I.

assumed a constant position. Most of the experiments were carried out with a preparation of relaxin prepared from sow ovaries[5] and injected intraperitoneally into the test animals.

RESULTS

To determine the extent of pubic separation occurring normally in pregnant mice, which could be determined by our method, a group of animals was followed throughout pregnancy. Our data confirmed the results of Hall and Newton (1946). During pregnancy, the pubic gap increased from 0.57 ± 0.13 to 4.27 ± 0.21 mm.; the most intense effect being found during the last six days of gestation. Within three days after delivery, the pubic gap decreased from 4.27 ± 0.21 to 2.47 ± 0.25. At eight days post-partum, the pubic gap was 2.07 ± 0.18.

Our attention turned to the actual assay of relaxin, and an effort was made to discover optimal conditions to develop a convenient, sensitive, reproducible assay technic. Accordingly a number of variables were investigated which are presented.

The timing of relaxin injections was first studied not only to discover the maximal pubic separation possible, but also the most efficient separation with the least hormone. A series of experiments was done using ovariectomized virgin mice in the weight range of 20–25 gm. Five days after surgery, 1 μg. estradiol benzoate was administered intraperitoneally daily for eight days. The eighth day of estrogen treatment was considered zero time for the injection of relaxin. Single daily doses were comparatively ineffective in maintaining pubic separation, even when repeated over a number of days. Radiographs taken at 2–4 hour intervals throughout the 24 hour period following the first injection indicated a maximum separation occurred at 16–18 hours.

Experiments were next performed where relaxin was injected at two hour intervals in four divided doses at 0, 2, 4, and 6 hours time in the hope that such a divided dose schedule would provide for a continued pubic separation. The course of the pubic separation resulting from such a dose regime was not different from that resulting from the administration of a single dose of relaxin. The doses of relaxin given at 2, 4, and 6 hours did not extend the peak of separation. Apparently the separation process began with the initial dose of relaxin and was not prolonged by the subsequent doses.

However when the time interval between doses was extended and injections given twice per day the separation became additive and radiographs taken at 24-hour intervals showed a progressive separation. When

[5] Chilcott "Releasin" #500 provided through the courtesy of Dr. Robert Kroc, Chilcott Laboratories. The Maltine Co., Morris Plains, N.J.

injecting the animals twice per day it was found that an 8–16 hour schedule was equivalent to a 12-12 hour schedule, and when injections were continued for periods up to nine days the maximum separation occurred at 2–5 days with increasing variable receptivity to relaxin. Therefore an assay period of 48 hours was selected as representing the time of optimal response. In an additional experiment the pubic response to four doses of relaxin administered at 12-hour intervals was compared to that of six doses given at 8-hour intervals. The data are presented in Figure 2. The total

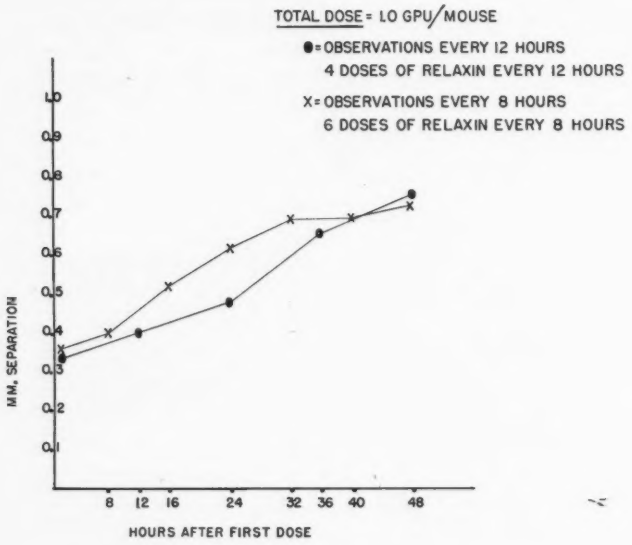

FIG. 2.

amount injected in each group was 1.0 G.P.U. per day and no significant difference was apparent. Therefore an 8-16-8-16 hour, twice per day for 48 hours, injection schedule for all assays was adopted.

In a few experiments, treating the mice with ACTH, cortisone, progesterone, or desoxycorticosterone in addition to estrogen and relaxin resulted in no increment in response. Control groups pre-treated with estrogen and injected with saline showed no response.

Before each assay, a control radiograph was taken immediately prior to the relaxin injection. The average pubic separation of both ovariectomized and intact, immature female mice, as determined after estrogen pretreatment but prior to relaxin administration, ranged from 0.32 to 0.35 mm. About 15% of the ovariectomized animals had an initial pubic separation of over 0.5 mm., and although experience showed that these partic-

ular animals responded with an equal or greater relative separation than the remaining animals, they were arbitrarily discarded in order to reduce assay variation.

The pubic responsiveness to relaxin was not influenced by the age at which ovariectomy was performed between 30 and 73 days of age. No significant difference in response could be detected between the following varying conditions: Administration of estrogens subcutaneously and of relaxin intraperitoneally, both hormones administered subcutaneously, and

TABLE 1. INFLUENCE OF NUMBER OF DAYS OF ESTROGEN PRETREATMENT ON PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:
Relaxin injections—2 per day for 2 days.
Observations—Pre-relaxin, 24 hours and 48 hours post-relaxin.
Estrogen—1 μg./day estradiol benzoate in corn oil.
Relaxin—0.5 G.P.U./day.

No. of estrogen inj. before relaxin treatment	No. of mice	Mean weight gm. ±S.E.	Pelvic separation		
			Pre-relaxin mm.	Post-relaxin 24-hrs. mm.	Post-relaxin 48-hrs. mm.
4	16	28 ±0.5	0.33 ±0.01	0.50 ±0.66	0.74 ±0.07
5	30	28 ±0.5	0.32 ±0.01	0.53 ±0.05	0.74 ±0.06
6	7	30 ±0.9	0.34 ±0.02	0.56 ±0.11	0.76 ±0.21
6	16	29 ±0.7	0.32 ±0.01	0.48 ±0.07	0.65 ±0.10
6	15	26 ±0.4	0.36 ±0.02	0.49 ±0.04	0.65 ±0.08
6	16	28 ±0.7	0.35 ±0.01	0.50 ±0.04	0.68 ±0.08
7	9	28 ±1.0	0.32 ±0.01	0.45 ±0.03	0.59 ±0.09
8	15	27 ±0.8	0.33 ±0.02	0.46 ±0.05	0.67 ±0.10

finally the estrogen administered intraperitoneally and the relaxin subcutaneously.

Estrogen pretreatment was varied from four to eight days as illustrated in Table 1. No significant difference in response could be observed.

An attempt was made to use the male mouse for the assay. As Table 2 illustrates, no significant pelvic response could be elicited even when doses as high as 1.2 G.P.U. per day were administered.

Attempts to establish dose-response curves are recorded in Tables 3 to 6. In Table 3 the response of increasing doses of relaxin from 0.15 G.P.U. to 0.60 G.P.U. per day was studied with an estrogen pretreatment period of six days. As little as 0.15 G.P.U. total dose produced a significant response in 24 hours, but when the dose within the day period, was increased to 0.30 or 0.60 G.P.U. only small increments in response were apparent. When the assay was run for two days, a relatively large difference between 0.15 G.P.U. per day and 0.30 G.P.U. per day was found. Increasing the dose to 0.60 G.P.U. per day in the two-day assay did not result in a significant increase in response. The data were also used in a

2×2 design according to Bliss (1944). Nine animals were used at each concentration of 0.15 and 0.30 G.P.U. per day. A potency ratio of 126.6% was found. The error at P=0.95 was +252% and −63%. The t value was 0.640 and the index of λ was equal to 0.426.

TABLE 2. MALE MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:									
Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R**	E R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 48 hrs. mm.
0.15	13	28 ±0.7	0.32 ±0.01	0.32 ±0.01	0.32 ±0.01
0.30	15	26 ±0.6	0.32 ±0.01	0.33 ±0.01	0.34 ±0.01
0.60	16	28 ±0.6	0.32 ±0.01	0.36 ±0.02	0.46 ±0.05
1.20	20	29 ±0.7	0.30 ±0.01	0.34 ±0.01	0.34 ±0.01

*E = Estradiol benzoate in corn oil 1 μg./day.
**R = Chilcott Releasin 31-81D SF.

TABLE 3. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule:									
Days	1	2	3	4	5	6	7	8	9
Treatment	E*	E	E	E	E	E	E R** R	E R R	
Observation							O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Day 7 pre-relaxin mm.	Day 8 post-relaxin 24 hrs. mm.	Day 9 post-relaxin 4-hrs. mm.
0.15	19	28 ±0.5	0.33 ±0.01	0.41 ±0.03	0.57 ±0.04
0.30	30	28 ±0.6	0.33 ±0.01	0.53 ±0.05	0.91 ±0.10
0.60	13	31 ±2.4	0.33 ±0.02	0.55 ±0.10	0.93 ±0.12

* E = Estradiol benzoate in corn oil 1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Nine.
X = 100 dose per day.
$Y = 1.16\ (X - 1.36) - 0.78$.
Doses from 0.15 to 0.30 G.P.U./day.

Additional dose-response data are presented in Tables 4, 5, and 6. In Tables 4 and 5, immature intact female mice were used in place of ovariectomized animals. When two doses of estrogen were employed daily (Table 5) a reasonably linear response was observed over the range 0.05 to 0.50 G.P.U. per day. With one dose of estrogen per day, the useful range was 0.05 to 0.25 G.P.U. per day.

Table 6 summarizes the dose-response data using ovariectomized females with an estrogen pretreatment period of five days. The slope of 0.60

is favorable and the useful range appears to be between 0.10 to 0.80 G.P.U. per day.

The utility of the method (see Table 6) was further tested by the simplified 2×2 design of Bliss. Two concentrations of each of the unknown

TABLE 4. RELAXIN IN IMMATURE, INTACT FEMALES (28 DAYS AT START)

Injection—observation schedule:

Days	1	2	3	4	5	6
Treatment	E*	E	E	E R**	E R	E
Observation				R O	R O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm. ±S.E.	Pelvic separation		Day 6 post-relaxin mm.
			Day 4 pre-relaxin mm.	Day 5 post-relaxin mm.	
0	8	16±0.9	0.34±0.01	0.34±0.01	0.34±0.02
0.05	13	18±0.5	0.35±0.01	0.40±0.02	0.38±0.03
0.10	14	17±0.4	0.32±0.01	0.48±0.04	0.51±0.06
0.25	17	16±0.7	0.36±0.01	0.53±0.05	0.61±0.05
0.50	18	16±0.6	0.33±0.01	0.47±0.03	0.56±0.05

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D SF.
Data of Day Six
$X = 100$ (dose per day).
$Y = 0.57\ (X = 1.07) + 0.51$.
Doses from 0.05 to 0.25 G.P.U./day.

TABLE 5. MOUSE PELVIC RESPONSE TO RELAXIN IN IMMATURE, INTACT, FEMALES (28 DAYS AT START)

Injection—observation schedule:

Day	1	2	3	4	5	6
Treatment	E* E	E E	E E	E+R** E+R	E+R E+R	
Observation				O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ±S.E.	Day 4 pre-relaxin mm.	Day 5 post-relaxin mm.	Day 6 post-relaxin mm.
0.05	21	17±0.4	0.32±0.01	0.34±0.01	0.37±0.01
0.10	18	17±0.8	0.32±0.01	0.40±0.03	0.50±0.06
0.25	20	17±0.04	0.33±0.01	0.53±0.05	0.71±0.07
0.50	40	17±0.04	0.35±0.01	0.69±0.06	0.84±0.08

* E = Estradiol aqueous.
** R = Chilcott Releasin 31-81D AF.
Data 6
$X = 100$ (dose per day).
$Y = 0.412\ (X - 1.30) + 0.65$.
Doses from 0.05 to 0.50 G.P.U./day.

and standard were 0.25 and 0.50 G.P.U. per day. Since the same material was used for both the unknown and the standard, the theoretical potency ratio was 100. When ten animals were used at each concentration for both unknown and standard a potency ratio of 77% was found. The t value for

significance of difference of slopes was 0.915 showing that the slopes were not significantly different, and at P = 0.95, the expected error determining the potency ratio was −49% and +96%. When the number of animals was increased to 16 in each group, the potency ratio was found to be

TABLE 6. MOUSE PELVIC RESPONSE TO RELAXIN

Injection—observation schedule								
Days	1	2	3	4	5	6	7	8
Treatment	E*	E	E	E	E	E R**	E R	
						R	R	
Observation						O	O	O

Amount of relaxin G.P.U./day	No. of mice	Mean weight gm ± S.E.	Pelvic separation		
			Day 6 prerelaxin mm.	Day 7 post-relaxin 24 hr. mm.	Day 8 post-relaxin 48 hr. mm.
0	21	29 ± 0.6	0.30 ± 0.01	0.33 ± 0.03	0.29 ± 0.01
0.10	17	27 ± 0.5	0.30 ± 0.01	0.33 ± 0.03	0.37 ± 0.05
0.25	19	26 ± 0.6	0.31 ± 0.01	0.42 ± 0.04	0.50 ± 0.05
0.50	30	28 ± 0.5	0.32 ± 0.01	0.53 ± 0.05	0.74 ± 0.06
0.60	21	30 ± 0.6	0.33 ± 0.01	0.49 ± 0.06	0.77 ± 0.09
0.80	12	31 ± 0.5	0.30 ± 0.01	0.76 ± 0.14	1.03 ± 0.13
1.00	7	28 ± 0.9	0.37 ± 0.02	0.66 ± 0.10	1.10 ± 0.20
4.00	9	28 ± 0.6	0.33 ± 0.02	0.87 ± 0.18	1.20 ± 0.24

* E = Estradiol benzoate in corn oil—1 μg./day.
** R = Chilcott Releasin 31-81D SF.
Data of Day Eight
$X = 100$ (dose per day).
$Y = 0.66\ (X - 1.57) + 0.68$.
Doses from 0.10 to 0.80 G.P.U./day.

91.5% and the error at P = 0.95 was −35 to +55%. The index of precision λ was equal to 0.375. The t test for differences between slopes was 0.673 which means that the slopes of unknown and standard were not significantly different.

DISCUSSION

One of the major advantages of employing mice for the bioassay of relaxin, aside from the obvious advantage of housing small animals, is that the animals are used only once and then discarded. In this way the sensitivity of the assay animal tends to be constant and the sensitizing effects of previous courses of estrogen and relaxin are avoided. The effect of employing the same animals for more than a single assay was effectively demonstrated when a group of nine animals were given the usual assay treatment of six days pre-treatment with estrogen plus two days estrogen and relaxin at 0.5 G.P.U. per day. The mean pubic separation increased from 0.33 mm. ± 0.02 to 0.86 mm. ± 0.15. After a five day rest period these animals were again used. This time the mean pubic separation was

0.84 ± 0.17 at zero hours and increased to 1.44 ± 0.24 at the end of 48 hours. This tendency to increased sensitivity to estrogen and relaxin following a previous pregnancy or assay use has been observed by various workers and represents a serious drawback in the conventional guinea pig assay where the animals are used repeatedly without a rest period of at least 7 to 10 days.

Talmage and Hurst (1950) attempted to refine the traditional guinea pig palpation assay by the radiographic measurement of the interpubic distance, but apparently the guinea pig pelvis separates less than 50 per cent over the resting gap as determined by these workers radiographically. According to their published figures the average interpubic distance determined before relaxin administration among their assay animals was between 0.75 and 1.0 mm. Following the injection of relaxin, there was an average increase of approximately 0.5 mm., but the great variability of response not only between individual animals, but between similar doses in the same animal, was so large that these observers concluded at least 50 animals at each of three dose levels would be necessary to provide a valid assay. They also observed a failure to return to the pre-relaxin measurement in about 10 per cent of pigs used for repeated assays, and a significant number of pigs repeatedly employed for relaxin assay sooner or later became permanently relaxed and had to be discarded.

The superiority of mice as compared to guinea pigs for relaxin assay is undoubtedly related to the dramatic separations which can be produced in the mouse by artificial means. Although we were unable to completely reproduce the separations observed in normal pregnant mice, this objective was more nearly attainable in mice than in guinea pigs. When 10 G.P.U. of relaxin were administered daily in two divided doses for eight days, the pubic gap increased from 0.29 mm. ± 0.02 to 2.49 ± 0.30. This was the maximum increment in pelvic gap produced by relaxin, and represented more than 800 per cent of the resting gap. It is probably true that the pubic separation occurring in pregnant mice undoubtedly represents an optimal hormonal environment culminating in a peak separation at term. Hall (1946) has shown that injections of estrone and/or progesterone into intact pregnant mice do not increase the observed separation.

In most of the experiments presented in this paper the mice were selected for assay on the basis of body weight. These animals were therefore of different ages and in order to determine the effect of age upon the individual sensitivity to relaxin, several experiments were performed with groups of animals of various known ages (30 to 73 days). No consistent effect attributable to age could be ascribed and at this time the effect of age prior to ovariectomy cannot be evaluated.

Recent work has been with unoperated intact immature female animals.

The pre-relaxin baseline separation in these animals, as compared with ovariectomized mice, was relatively more constant and only an occasional animal was discarded because the gap measured over 0.5 mm. This assay technique is apparently practical and although the maximum response seems less than with ovariectomized animals, an attempt is being made to determine its possible value.

SUMMARY

A sensitive, convenient and practical bioassay for relaxin is presented employing mice, with radiographic measurement of the pubic separation.

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BIOCHEMICAL PROPERTIES OF FRACTIONS OBTAINED FROM RAT ANTERIOR PITUITARY GLANDS BY DIFFERENTIAL CENTRIFUGATION[1]

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THE biochemistry of the nuclear, mitochondrial, microsomal and supernatant fractions isolated by differential centrifugation from rat and mouse liver has been studied extensively during the past few years. Particular attention has been given to the distribution of the oxidative enzymes and nucleic acids among these fractions. Respiratory enzymes such as cytochrome oxidase and the succinoxidase system have been found to be associated with the mitochondrial fraction (Schneider, 1946; Hogeboom *et al.*, 1946 and 1948). The microsomal fraction contains few enzymes but as much as 50% of the pentosenucleic acid of liver tissue (Hogeboom *et al.*, 1948; Schneider *et al.*, 1950.) The results of studies of this nature have been summarized by Schneider and Hogeboom (1951).

Little attention has been given to the biochemical and hormonal properties of fractions of endocrine tissues isolated by differential centrifugation. Approximately 60% of the succinoxidase activity of rat anterior pituitary glands, however, has been reported to be associated with a large granule fraction (McShan and Meyer, 1949), and 75% of the gonadotrophic hormone activity with a small granule fraction (McShan and Meyer, 1952). The purpose of this paper is to describe a study of the gonadotrophic hormone activity of fractions obtained from anterior pituitary glands of adult normal and castrate rats as related to the succinoxidase, glycolytic and transaminase activities; the desoxypentose-nucleic acid (DNA) and pentosenucleic acid (PNA); and the nitrogen or protein contents of these fractions.

MATERIALS AND METHODS

The anterior pituitary glands of normal and castrate adult female rats of the Holtzman-Rolfsmeyer strain were used for fractionation. The minimum time of castration before using the animals as donors was three weeks and the maximum time was six months.

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The use of rats for experiments of this kind is advantageous as the glands can be removed and homogenized within a few minutes after the animals are killed. This allows little time for tissue changes to occur.

Groups of 100 to 150 rats were used for each run. The pituitary glands were removed in the cold room immediately after killing the animals, and kept in a humidor during the short time required to complete removal of all the glands. The anterior lobes were separated, weighed and macerated in a sharp pointed homogenizing tube with 0.88 M sucrose. The volume of the sucrose in milliliters was 19 times the weight of the glands in grams. The homogenates were prepared by mashing the glands instead of grinding since less gonadotrophin is found in the supernatant fraction when the former procedure is followed (McShan and Meyer, 1952).

The homogenates (WH) were differentially fractionated in the multispeed head of an International refrigerated centrifuge at 2° C. by centrifuging at 275, 4,400 and 20,400 g. to give, respectively, the nuclear (NF), large granule (LG), small granule (SG) and supernatant (S3) fractions according to the procedure of McShan and Meyer (1952). The saline extract of the SG fraction was indicated by SGE and the extracted granules by SGR.

The homogenates and fractions were assayed for gonadotrophic hormone activity by use of 21 day old female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with normal saline so that each rat received a total of 20 mg. equivalent of fresh tissue in nine injections of 0.5 ml. each. The first injections were made on the afternoon of the first day followed by injections on the morning and afternoon of the next four days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded.

The homogenates and the fractions were stained for mitochondria by use of fast green FCF as reported by Harman (1950) and the nitrogen determinations were done by a modification of the micromethod reported by Johnson (1941).

The procedure for the preparation of filtrates for the nucleic acid determinations was based on the method reported by Schneider (1945a). The exact procedure employed was as follows: The sucrose homogenates and fractions were mixed with one-half volume of 20% trichloracetic acid (TCA), centrifuged at 2,500 RPM in the cold (2° C.) and the supernatant liquid decanted. The precipitates were suspended in 1 to 2 ml. of water to which was added an equal volume of 10% TCA followed by centrifugation. The supernatant liquid was decanted and the precipitates were washed once more with 5% TCA.

The above precipitates were suspended in 1 ml. of water to which was added 3 ml. of 95% ethyl alcohol. This mixture was centrifuged at room temperature followed by decantation of the supernatant liquid, and a second washing with alcohol.

The alcohol precipitates were mixed with 3 ml. of 3:1 ethyl alcohol (95%)—ether mixture, centrifuged and the supernatant liquids decanted. The precipitates were mixed with 2 to 5 ml. of 5 per cent TCA depending on the amount of tissue and heated at 90° C. for 15 minutes, centrifuged and the clear TCA extracts decanted into graduated 15 ml. centrifuge tubes. The precipitates were washed once with 1.5 ml. of 5% TCA, and centrifuged. The washings were mixed with the above extracts and the total volumes were recorded.

The PNA content of the above TCA extracts was determined by the orcinol method of Mejbaum (1939) as recommended by LePage and Umbreit (1945). DNA was determined by the diphenylamine reaction of Dische (1930) as modified by Schneider (1945).

As a basis for calculating the amounts of DNA and PNA present in the homogenized tissues and fractions certain analytical constants for purified samples of these acids were determined according to Schneider (1945a, 1945b). Constants found for the Beckman Model DU spectrophotometer were Fa = 0.01878 (diphenylamine), Fo = 0.1344 (orcinol), and C = 0.01216. These constants were used for calculating the results since the determinations were made with the Beckman instrument. The percentage values used for phosphorus were 0.0954 for PNA and 0.0992 for DNA. A sample calculation using constants for the Evelyn colorimeter is given in the report by McShan, *et al.* (1950).

The succinoxidase was determined by the method of Schneider and Potter (1943), anaerobic glycolytic activity by the method of Novikoff, Potter and LePage (1948), and transaminase activity by the method of Ames and Elvehjem (1946) as modified by Copenhaver, Shipley and Meyer (1951).

RESULTS AND DISCUSSION

The results of the biochemical studies of anterior pituitary tissue from normal and castrate female rats are summarized in Table 1. The gonadotrophic hormone activity of glands from castrate rats was much greater than that of those from normal animals, and the major part of the activity of both kinds of glands was recovered in the small granule (SG) fraction. Essentially all the gonadotrophin in the SG fraction of castrate females was removed by extracting eight times with 0.9% NaCl solution. This extract was designated as SGE. The extracted granules (SGR), and the NF and LG fractions contained little activity whereas the final supernatant fraction S3 contained gonadotrophic activity sufficient to produce ovaries that had an average weight of 55 mg.

Essentially all the DNA of the glands from normal and castrate rats was recovered in the nuclear fraction (NF). The DNA per whole gland was 71.2 μg. for normal and 70.0 μg. for castrate glands (Table 2). On the basis of the assumption that the DNA content of the pituitary cells is constant (Boivin *et al.*, 1948; Swift, 1950), the castrate glands contained about the same number of cells per unit weight as the normal glands. The average weight of the anterior pituitary glands was 10.4 mg. for normal and 11.1 mg. for castrate rats. The ratio of gland weight to weight of DNA per gland was 146 for normal and 159 for castrate animals. This ratio is an expression of relative cell weight and, on the assumption that the specific gravities are the same, it indicates that at least some cells of the castrate glands were larger than those of normal glands. This is supported by the well known fact that the basophils undergo hypertrophy after castration.

The protein values are given in terms of mg. of protein per 20 mg. of fresh tissue (Table 1). These values for protein (N × 6.25) are equivalent to the total nitrogen of the various fractions and are, therefore, maximum values since a small amount of nonprotein nitrogen was presumably present. The protein value for WH of glands from normal rats was 15% greater than was that for castrate animals. The values for the LG, SG and

TABLE 1. BIOCHEMICAL PROPERTIES OF FRACTIONS OBTAINED FROM HOMOGENATES OF NORMAL AND CASTRATE ADULT FEMALE RATS

Activity or compound	Units (mg. FT)[1]	Kind of rats[2]	Pituitary fractions								
			WH	NF	LG	SG	SGR	SGE	S3	RCF[3]	Recovery %
Assay—av. wt. ovaries (mg.)[4]	20 20	N C	32 (9)[5] 201(18)	35(6)	36(15)	25 (9) 159(15)	25(17)	145(21)	55(15)		
Protein (N×6.25)	mg./20 mg.	N C	3.56(4)[6] 3.05(6)	1.03(4) 1.01(6)	0.38(4) 0.30(6)	0.54(4) 0.45(4)	0.23(4) 0.20(6)	0.27(2) 0.21(6)	1.62(4) 1.56(6)		100 109
Nucleic acid PNA	mg./100 gm.	N C	343.6(4) 369.4(6)						155.8(5) 150.0(6)		
DNA	mg./100 gm.	N C	685.2(5) 630.4(5)	691.0(6) 602.2(6)							101 96
Glycolysis	cmm. CO_2/ 20 mg./10 min.	N C	25.0(3) 21.3(3)	7.0(3) 5.9(3)	2.6(3) 3.1(3)	1.4(3) 2.1(3)			9.5(3) (9.8(3)	17.2(3) 21.9(3)	82 103
Transaminase	cmm. CO_2/ 20 mg./10 min.	N C	162.0(1) 149.4(4)						116.0(1) 125.4(4)		
Succinoxidase	cmm. O_2/ 20 mg./10 min.	N C	16.3(3) 14.3(5)	3.9(3) 2.7(5)	4.2(3) 6.5(5)	5.4(3) 1.8(4)			0.23(3) 0.50(4)	16.6(3) 15.3(4)	102 107

[1] Milligrams of fresh tissue unless indicated otherwise.
[2] N, normal; C, castrate rats.
[3] Recombined fractions.
[4] Average weight of ovaries from untreated animals of the same age is 13 mg.
[5] Number of animals used for assays.
[6] Number of determinations on which the average value is based.

TABLE 2. DESOXYPENTOSE NUCLEIC ACID CONTENT OF NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Kind of rats	Normal	Castrate
DNA per 100 gm. tissue (mg.)	685.2(5)[1]	630.4(5)
Weight of gland (mg.)	10.4(768)[2]	11.1(958)
DNA per gland (μg.)	71.2	70.0
Gland weight (μg.)/DNA per gland (μg.)	146.0	159.0

[1] Number of determinations on which average value is based.
[2] Number of rats on which average is based.

SGE fractions of glands from normal animals were slightly greater than the values for these fractions of glands from castrate rats. More extractable protein (SGE) was associated with the SG of normal than with those of castrate glands, but the SGR fractions of both normal and castrate glands contained essentially the same amount of protein, namely, 0.23 mg. and 0.20 mg., respectively. Results given in Table 3 show that the protein per gland and the relative amount per cell recovered in the LG, SG and SGE fractions were slightly greater for normal than for castrate glands even though the SG and SGE fractions from the castrate glands contained much more gonadotrophic activity. This means that per unit weight of protein the small granule fraction of glands from castrate rats was much richer in gonadotrophin than the same fraction from glands of normal animals.

The fact that only a slight increase occurred in the total PNA on castration (Table 1) indicates, according to Caspersson (1947), little increase in pituitary protein during castration. Synthesis of small amounts of protein for purposes such as the production of gonadotrophin could have conceivably occurred. However, the possibility should not be overlooked that on castration gonadotrophin was formed from protein already present. It is not clear, of course, as to whether the gonadotrophin was produced by the particules with which it was associated or was concentrated in these particles after production at some other point in the cell.

Essentially 50% of the PNA of the homogenates was recovered in

TABLE 3. CONCENTRATIONS OF PROTEIN IN FRACTIONS FROM NORMAL AND CASTRATE RAT ANTERIOR PITUITARY GLANDS

Compound	Kind of rats	Fraction						
		WH	NF	LG	SG	SGR	SGE	S3
Protein per gland (μg.) (N×6.25)	N	1850	540	198	280	120	140	840
	C	1690	560	166	250	116	115	866
Protein per gland (μg.)[1]	N	26.0	7.58	2.78	3.93	1.72	1.97	11.80
DNA per gland (μg.)	C	24.7	8.00	2.40	3.60	1.66	1.64	12.40

[1] Relative measure of protein per cell.

the S3 fraction. PNA was also found in each of the other fractions but these data are not considered to be reliable from the quantitative standpoint because small amounts of tissue were used for the determinations and the recoveries were not satisfactory. These data are not included. The PNA values for the homogenates were lower than found previously for aqueous homogenates of pituitary glands from young adult rats.

The glycolytic activity of the homogenates of glands from normal rats was slightly greater than the activity of those of castrate rats (Table 1). The NF of both kinds of glands contained more activity than the LG and SG fractions. Almost 50% of the glycolytic activity was found in the supernatant fraction S3 of both the normal and castrate glands which is similar to the results reported by LePage and Schneider (1948) for the soluble fraction of rabbit liver. The SG fractions from both normal and castrate glands were low and not significantly different in glycolytic activity whereas these fractions from normal glands were low and those from castrate glands were high in gonadotrophic activity.

A high percentage of the transaminase activity from both kinds of glands was recovered in the S3 fraction.

The succinoxidase activity of the homogenates of normal glands was 16.3[2] as compared with 14.3 for castrate glands. Satisfactory recovery of activity was obtained when the various fractions were recombined as shown by values of 16.6 and 15.3, respectively, for normal and castrate glands. The NF, LG and SG fractions obtained from both kinds of glands by the procedure referred to above (1952) contained succinoxidase activity. This suggested that these fractions also contained mitochondria and this was shown to be the case by staining. The number present in each of these fractions was not estimated quantitatively. The impression was gained from the staining that mitochondria of varying sizes were present. The fact that they were found in the fractions recovered at different speeds of centrifugation is also indicative of this.

The succinoxidase value for the SG fraction of normal glands was three times the value for castrate glands, namely, 5.4 to 1.8 (Table 1). The latter value for glands of castrate rats is an average of three very low and one high value. The difference between these two values for succinoxidase activity is probably not of any particular significance.

The fact that the LG fraction contains little gonadotrophic activity indicates that gonadotrophin is not associated to a significant degree with the mitochondria of this fraction. On the basis of this it was suggested (McShan and Meyer, 1952) that the gonadotrophin of the SG fraction was not associated with the mitochondria of this fraction, but this explanation

[2] Cu. mm. of oxygen uptake per 20 mg. of fresh tissue per 10 minutes.

does not seem valid since it is conceivable that the gonadotrophin could be associated with the small mitochondria or with other granules contained in the SG fraction. It was reported previously (McShan and Meyer, 1952) that little gonadotrophin is associated with the material recovered from the S3 fraction of an 0.88 M sucrose homogenate by centrifuging at 150,000 g. which, according to the work of Hogeboom, Schneider and Pallade (1948), would be expected to contain submicroscopic particles.

SUMMARY AND CONCLUSIONS

Anterior pituitary glands from normal and castrate adult female rats were homogenized in 0.88 M sucrose and separated by differential centrifugation into nuclear, large granule, small granule and supernatant fractions. The gonadotrophic activity, the protein, PNA and DNA contents, and the glycolytic, transaminase and succinoxidase activities of the homogenates and fractions of both kinds of glands were studied.

The high level of gonadotrophin found in the castrate as compared with the normal glands was concentrated in the small granule fraction, and it was removed from this fraction by extraction with isotonic saline. The distribution of protein in the two kinds of glands was studied. The granule fractions and the extract of the small granules of the castrate glands contained slightly less protein than the same fractions of normal glands.

The DNA was recovered in the nuclear fraction and approximately 50 per cent of the PNA in the supernatant fraction. Castration caused little change in the DNA and PNA.

The glycolytic activity was essentially the same for the homogenates and fractions obtained from glands of normal and castrate rats, but the gonadotrophic hormone activity was much greater in the small granule fraction of glands from castrate as compared with normal animals. The major part of the transaminase was found in the supernatant fraction. The succinoxidase was distributed in the nuclear and granule fractions of both kinds of glands. These fractions as indicated by staining were found to contain mitochondria.

On the basis of these results and those reported previously it appears that though gonadotrophin is not associated to a significant degree with the mitochondria of the large granules nor with the submicroscopic particles recovered by high speed centrifugation of the supernatant fraction, it may be associated with those of the small granules.

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RESPONSE OF PREPUTIAL AND ADRENAL GLANDS OF HYPOPHYSECTOMIZED RATS TO ACTH[1]

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THE growth response of the preputial glands of the female rat to ACTH in adrenalectomized-ovariectomized animals indicates that these glands are subject to the direct action of this trophic hormone (Jacot and Selye, 1952). Furthermore, it has been shown that the ascorbic acid of male rat preputial glands can be depleted by stress or ACTH in acute experiments, and that these alterations in the vitamin content resemble the changes which occur in the adrenal gland following these same stimuli (Hess *et al.*, 1952).

It has been known for some time that the level of adrenal ascorbic acid is greatly reduced by hypophysectomy (Leblond and Chamorro, 1940; Bowman *et al.*, 1940). Tyslowitz (1943) reported that the concentration of ascorbic acid in the adrenal glands gradually declines for about 18 days following the operation. Thereafter, he found that the concentration remained stabilized at about 60% of the value for intact animals. To date, there is no information concerning the concentration of ascorbic acid in the preputial glands of the hypophysectomized rat. The present studies were undertaken to determine the effect of hypophysectomy, together with acute and chronic administration of ACTH, on the weight and ascorbic acid content of the preputial glands, as compared to the adrenal glands of the rat.

METHODS

Twenty-nine male rats of the Holtzman strain were hypophysectomized at the age of 33 days. During the postoperative period they were maintained on laboratory chow and 5% sucrose solution. At the time of autopsy the sella turcica was examined for hypophyseal fragments with the aid of a dissecting microscope. The absence of any such fragments, together with a sustained loss of body weight and a marked loss of testis and ventral prostate weight was considered satisfactory evidence of the completeness of the operation.

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Experiment I. At the end of 3 weeks following hypophysectomy the left preputial and left adrenal glands were removed from 14 animals under nembutal anesthesia. Ten of the animals were then injected with 10 μg. ACTH,[3] via the tail vein, and after one hour the right preputial and right adrenal glands were removed. The glands were freed of excess adipose and connective tissue, weighed on a torsion balance, and subjected to ascorbic acid analysis in accordance with the method of Roe and Kuether (1943).

Experiment II. Two weeks after hypophysectomy, the left preputial and left adrenal glands were surgically removed from 15 animals. Following this operation, the animals were given subcutaneous injections of 25 μg. ACTH twice daily for 7 days. At autopsy the right preputial and right adrenal glands were removed, weighed and analyzed for ascorbic acid content.

Twenty-three intact animals of the same age and sex were used as controls for these experiments. In addition to weights and ascorbic acid values for preputial and adrenal glands, weights for the left testis and ventral prostate were obtained from these animals.

RESULTS

Experiment I. Three weeks after hypophysectomy there is an absolute as well as a relative decrease in the weight and ascorbic acid content of the preputial and adrenal glands (Table 1). On a relative weight basis the

TABLE 1. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE ADRENAL AND PREPUTIAL GLANDS OF MALE RATS

	No. of Rats		Adrenal Glands				Preputial Glands			
			Weight		Ascorbic Acid Content		Weight		Ascorbic Acid Content	
			Abs	Rel.[2]	Abs.[3]	Rel.[4]	Abs.	Rel.[2]	Abs.[3]	Rel.[4]
			mg.	mg.			mg.	mg.		
Control	10	L	13.9 ±0.30*	8.1 ±0.26	60 ±2.6	439 ±18	39.3 ±5.0	22.7 ±2.8	22 ±3.3	57 ±6.3
	10	R	13.1 ±0.22	7.6 ±0.20	60 ±2.2	456 ±16	32.4 ±4.4	18.6 ±2.4	20 ±3.1	59 ±6.8
Hypophysect. (3 weeks)	14	L	5.4 ±0.18	5.1 ±0.19	13 ±0.6	247 ±11	9.7 ±0.5	9.1 ±0.5	3 ±0.9	24 ±7.2
	10	R[5]	4.8 ±0.22	4.4 ±0.20	8 ±1.0	170 ±20	10.5 ±0.9	9.8 ±0.9	1 ±0.3	6 ±2.8
Hypophysect. (2 weeks)	15	L	5.6 ±0.19	5.3 ±0.25	19 ±1.7	348 ±26	11.0 ±0.7	10.5 ±0.7	3 ±1.1	21 ±9.1
Hypophysect. (3 weeks) plus ACTH[1]	15	R	6.1 ±0.28	5.8 ±0.30	17 ±0.8	276 ±12	15.4 ±0.9	14.5 ±0.8	4 ±1.6	29 ±10.3

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
[3] μg. of ascorbic acid in the entire gland.
[4] μg. ascorbic acid/100 mg. tissue.
[5] 10 μg. ACTH injected intravenously 1 hr. prior to autopsy.
* Standard error.

[3] Armour ACTHAR. (dosage expressed as LA-1A equivalent)

preputial and adrenal glands of the hypophysectomized rat are 60% and 37% smaller, respectively, than those of control animals. Likewise, the concentration of preputial and adrenal ascorbic acid is 58% and 44% less than that of the normal glands. In spite of the fact that the concentration of ascorbic acid is greatly reduced in these glands following hypophysectomy, they are still capable of responding to an intravenous injection of 10 μg. of ACTH by a further reduction in their ascorbic acid content (Table 1). Thus a 75% depletion of preputial and a 31% depletion of adrenal ascorbic acid was seen at one hour following the injection of ACTH.

Experiment II. The 7-day treatment with ACTH resulted in some enlargement of the preputial glands as seen by comparing their weights with those of uninjected hypophysectomized rats (Table 1). Likewise, there was a slight enlargement of the adrenal glands, although it was considerably less than was anticipated. It is clear that ACTH reverses the gradual decline in the weight of the preputial and adrenal glands seen at 2 and 3 weeks after hypophysectomy. These injections, on the other hand, had only little effect on the preputial and adrenal ascorbic acid concentrations, the values being similar to those observed in the uninjected, hypophysectomized animals.

It has been the general observation in this study that left preputial and adrenal glands are slightly heavier than right ones. This was true in the intact control group, where on a mg./100 gm. body weight basis, the left preputial gland was 18% heavier than the right and the left adrenal gland was 6% heavier than the right (Table 1). There was no significant difference, however, in the concentration of ascorbic acid in the right and left glands. In the two hypophysectomized groups, since the right glands were utilized for study after the administration of ACTH, either acutely or chronically, it is not known whether this same tendency prevails. If one assumes that left glands tend to be heavier than right ones in untreated, hypophysectomized rats, it follows that in the present study the preputial and adrenal hypertrophy produced by chronic ACTH administration is partially masked.

DISCUSSION

Following hypophysectomy there is a gradual decline both in the weight and ascorbic acid content of the preputial and adrenal glands. The results for the adrenal glands are in general agreement with the earlier findings of Tyslowitz (1943). The decline proceeds at a more rapid rate in the preputial glands where it is apparently complete at 2 weeks after hypophysectomy. It is of considerable interest that, in spite of the marked involution of the preputial and adrenal glands and their low levels of ascorbic acid, they are still sensitive to ACTH, as indicated by the further depletion of ascorbic acid following the acute administration of 10 μg. ACTH. In com-

parison to the adrenal gland, the preputial gland is apparently more sensitive to the ascorbic acid depleting action of ACTH.

Although the administration of ACTH for one week to animals which had been hypophysectomized 2 weeks previously was capable of reversing the gradual decline in preputial and adrenal weight, it had little effect in rebuilding the ascorbic acid content of these glands. The concentration of preputial and adrenal ascorbic acid is not significantly different from the values for the untreated, 3-week hypophysectomized animals. In a previous study of this sort Munson and Koch (1946) reported that 13 daily injections of ACTH to hypophysectomized rats raised the ascorbic acid content of their adrenal glands. Their dosage range was from 40 μg. to 1 mg. daily and they state that even when sufficient ACTH was given to increase the adrenal weight above that of unoperated controls, the adrenal ascorbic acid was still significantly less than that of the controls. It seems clear that the action of ACTH on adrenal as well as on preputial gland ascorbic acid is more complex than is indicated by the depletion phenomenon which rapidly follows the administration of a single injection. In the absence of further information it is suggested that ACTH may also influence the synthesis of ascorbic acid so that with the chronic administration of this hormone to hypophysectomized animals a new equilibrium between synthesis and utilization of ascorbic acid in these glands is reached. If this is true, it appears that at the dosage employed the activity level is well below that of the glands of intact animals, as indicated by the relatively low levels of glandular ascorbic acid. Further indication of low activity level is the relatively slight effect on the preputial and adrenal gland weight produced by the dosage of ACTH employed. Apparently the dosage of ACTH employed was near the minimally effective dosage of the preparation used.

It has been known for some time that the preputial glands of the rat are subject to androgenic stimulation (Korenchevsky, 1935). That this is not a factor in the present study is indicated by the pronounced atrophy of the testes and ventral prostate in both groups of hypophysectomized animals (Table 2). The preputial glands are also responsive to some degree to the action of estrogen and progesterone (Van der Woerd, 1938) and are currently regarded as accessory reproductive glands. Developmentally they are modified sebaceous glands and functionally they belong to the holocrine type, their secretory product being a viscous sebum. By usual histological and cytological criteria they have little in common with the adrenal cortex. It is therefore somewhat surprising that these glands show such a striking parallelism to the adrenal glands in their response to ACTH. So far as is known at present, the direct action of ACTH is limited to the adrenal cortex and the preputial glands. Long (1947) has shown that changes in ascorbic acid levels of other organs such as brain, kidney, heart,

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY AND ACTH TREATMENT ON THE BODY, TESTIS, AND PROSTATE WEIGHT OF RATS

	No. of Rats	Body weight		Left testis weight		Ventral prostate weight	
		Initial	Final	Abs.	Rel.[2]	Abs.	Rel.[2]
		gm.	gm.	mg.	mg.	mg.	mg.
Control	13	120 ±0.9*	179 ±2.9	1114 ±34	628 ±16	128 ±18	71 ±8.9
Hypophysect. (3 weeks)	14	122 ±1.7	109 ±1.8	206 ±12	191 ±11	9.5 ± 0.6	8.8 ±0.5
Hypophysect. (3 weeks) plus ACTH[1]	15	123 ±1.3	106 ±1.5	202 ±15	189 ±13	9.1 ± 0.8	8.6 ±0.8

[1] 25 μg. ACTH injected subcutaneously twice daily for 7 days prior to autopsy.
[2] mg./100 gm. body weight.
* Standard error.

skeletal muscle and lymphoid organs are of an insignificant nature following a single injection of ACTH. The marked differences together with the marked similarities between the preputial and adrenal glands offer the promise that further studies of this nature will shed new light on the role of ascorbic acid in cellular processes.

SUMMARY

Hypophysectomy of male rats produces a gradual decline in both weight and ascorbic acid content of the preputial and adrenal glands. At three weeks postoperatively when the concentration of ascorbic acid is at a low level in both of these glands it can be further reduced by a single intravenous injection of ACTH. A 7-day treatment, begun two weeks postoperatively reverses the gradual decline in the weight of the preputial and adrenal glands but has little effect on their ascorbic acid content. As in intact animals, there is a close parallelism in the response of the preputial and adrenal glands to ACTH.

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ENDOCRINE INFLUENCE ON THE PLASMIN-PLASMIN INHIBITOR SYSTEM IN THE BLOOD OF RATS

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INTRODUCTION

EARLIER studies have indicated that the equilibrium of the plasmin (fibrinolysin)-plasmin inhibitor (antifibrinolysin) system in blood can be shifted under various experimental conditions (Jensen, Chamovitz, Volkringer, Gray, Gosney and Kocholaty, in press). Ungar and his associates (1951, 1951) reported that certain endocrine principles act on this system by accelerating the rate of combination of plasmin and plasmin inhibitor. The findings of the present study do not seem to substantiate this concept. Instead, the recorded data tend to show that the increased antifibrinolytic activity, observed after administration of certain hormones, is a result of an actual rise in the plasmin inhibitor level in the blood.

MATERIALS AND METHODS

Male Sprague-Dawley albino rats weighing 220–250 gm were used. The animals were allowed food and water throughout the experiment. Unless noted all injections were given subcutaneously. Controls received either no injections or a volume of solvent equal to that used in the hormone injections.

Splenectomy was performed on fasting rats anesthetized by ether. Animals regained their normal weight within the second or third post-operative day. The experimental work was carried out 7 days after splenectomy. Hypophysectomized and adrenalectomized rats, obtained from the Hormone Assay Laboratories, Inc., Chicago, Illinois, were used 10 days after operation.

The hormones were injected subcutaneously in dosages as follows:

Adrenocorticotropin, Armour Acthar, equivalent to 1.0 mg Standard LA-1-A/kg twice daily for 3 days; controls—equivalent volume of 0.9% saline.

Cortisone, Merck Cortone Acetate, 5 mg/kg daily for 3 days; controls—equivalent volume of 0.9% saline.

Thyroxine, Squibb Crystalline Thyroxine, 0.5 mg/kg daily for 4 days; controls—equivalent volume of slightly alkaline 0.9% saline.

Thyrotropin,[1] Armour Lot 2-D-3, LJW3-16-48 containing 5 Junkmann-Schoeller units per mg, 10 mg/kg daily for 5 days; controls—equivalent volume of slightly alkaline 0.9% saline.

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[1] This preparation was kindly supplied by the Armour Laboratories, Chicago, Illinois.

Blood samples, 1.8 ml, were taken by heart puncture 2 hours after the last injection, using 0.2 ml of 3.8% sodium citrate as the anticoagulant. Following centrifugation at 2800 rpm for 30 minutes at 5 to 10°C, the plasma was separated and either analyzed immediately or stored frozen until tested.

The antifibrinolytic activity of plasma was determined by measuring the residual activity of a standard amount (2 mg[2]) of bovine plasmin,[3] after incubation for 5 and 30 minutes with a 4% concentration of plasma as previously described by Westphal, Kocholaty, McDonald, King and Jensen (1950). Thrombin, salt-free, (Parke-Davis) and fibrinogen, prepared according to Ware, Guest and Seegers (1947), were used. Calculations of antifibrinolytic units (AFU) were performed as previously reported by Kocholaty, Ellis and Jensen (1952).

The plasmin inhibitor preparation used in these studies was obtained by $(NH_4)_2SO_4$ fractionation of a product prepared from bovine plasma according to Loomis, Ryder and George (1949). The plasmin inhibitor was found to be precipitated between 0.6–0.7 saturation of ammonium sulfate.

RESULTS AND DISCUSSION

When the plasmin inhibitor preparation was tested against plasmin, it was found that 340γ of this preparation were equivalent to one anti-

TABLE 1. INHIBITION OF BOVINE PLASMIN BY NORMAL RAT PLASMA

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml of rat plasma ranging from 0.5–6% in the final reaction mixture. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ), the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasma concentration (%) in the reaction mixture	CDT (Seconds)*	
	5 minute incubation	30 minute incubation
0.0	123	126
0.5	129	136
1.0	135	147
1.5	145	160
2.0	153	175
2.5	166	195
3.0	182	216
3.5	203	249
4.0	234	305
4.5	275	355
5.0	314	420
5.5	382	528
6.0	455	610

* Each clot dissolution time (CDT) is the average of duplicate determinations.

fibrinolytic unit. Tested against trypsin, 60γ corresponded to one unit. This finding is in agreement with earlier observations of Christensen and MacLeod (1945) and of Lewis and Ferguson (1950) who found that the inhibition of plasmin requires a higher concentration of the serum or plasma than is necessary to inhibit trypsin of equal fibrinolytic potency.

[2] This amount of plasmin dissolved the standard fibrin clot in 125 seconds at 37.5°C.

[3] Preparations of bovine plasmin were kindly supplied by Dr. E. C. Loomis of the Research Laboratories, Parke-Davis and Company, Detroit, Michigan.

The rate of combination between plasmin and its inhibitor is dependent on the time of incubation of the reactants; a 30 minute incubation produced a greater inhibition than did the 5 minute incubation, both in the case of rat plasma and of a purified preparation of bovine plasmin inhibitor (Table 1 and 2). These findings are in agreement with similar observations reported by Guest, Daly, Ware and Seegers (1948). It was found that the inhibition of trypsin by normal rat plasma after 30 minutes incubation was somewhat greater than after 5 minutes incubation, but the

TABLE 2. INHIBITION OF BOVINE PLASMIN BY PLASMIN INHIBITOR

0.1 ml (2000γ) of bovine plasmin incubated for 5 and 30 minutes at 25° C. with 0.1 ml containing 20–140 of purified bovine plasmin inhibitor. After addition of thrombin (0.1 ml, 10 units) and fibrinogen (0.2 ml, 500γ) the clot dissolution time was determined at 37.5° C. Imidazole buffer pH 7.25 was used.

Plasmin inhibitor Gamma	CDT (seconds)*	
	5 minute incubation	30 minute incubation
0	126	129
20	130	138
40	131	145
60	134	154
80	137	163
100	139	166
120	142	177
140	143	193

* Each clot dissolution time (CDT) is the average of duplicate determinations.

difference was not as pronounced as that observed with plasmin. As to be expected, the rate of combination between plasmin and its inhibitor was found to be dependent also on the temperature of the reaction mixture.

The antifibrinolytic activity of normal rat plasma after 5 minutes incubation was 87.6 ± 7.5 units, after 30 minutes, 105.8 ± 5.8 units (Table 3). The plasmas of control rats injected either with saline or alkaline saline had similar antifibrinolytic activities. The administration of adrenocorticotropin over a 3-day period in normal rats produced a significant increase in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation, 101.4 ± 13.5 units and 119.3 ± 8.5 units, respectively, (Table 3). Thyrotropin, given for 5 days, produced an increase noted only after 5 minutes incubation. Thyroxine, administered for 4 days, produced an elevation of the antifibrinolytic activity which was evident only after 30 minutes incubation while cortisone, given for 3 days, produced no change.

Single intraperitoneal injections of adrenocorticotropin (1 to 4 mg. per rat), of adrenal cortical extract, Upjohn, (2 to 4 ml. per rat) and of thyroxine (1 mg. per rat) were found to produce no significant change in the plasmin inhibitor titer 15 to 90 minutes after administration.

The antifibrinolytic activity of plasma 10 days after removal of the hypophysis or of the adrenals was found to be greatly reduced (Table 3). Splenectomy did not alter the plasmin inhibitor level of plasma (Table 3). In splenectomized rats, adrenocorticotropin administration (same procedure as in normal animals) did not produce any change in the antifibrinolytic activity of the blood, as measured after 5 or 30 minutes incubation (Table 3). Apparently, the influence of the adrenals on the plasmin inhibitor titer is mediated through the spleen. In this connection it may be pointed out that Macfarlane and Biggs (1948) have observed that among all the organs, extracts of the spleen had the highest antifibrinolytic activity. On the other hand, administration of thyroxine (same procedure as in normal animals) to the splenectomized rat produced a significant elevation of the antifibrinolytic titer as measured after 30 minutes incubation (Table 3). Apparently, more than one mechanism is involved in the control of the plasmin inhibitor level in blood.

TABLE 3. EFFECT OF REMOVAL OF ENDOCRINE ORGANS AND OF VARIOUS HORMONES ON THE ANTIFIBRINOLYTIC ACTIVITY OF RAT PLASMA

Treatment	No. of animals	Incubation time: 5 Minutes			30 Minutes		
		CDT[1] Avg. Sec.	AFU[2]/ml plasma Avg. ±S.D.	t[3]	CDT Avg. Sec.	AFU/ml plasma Avg. ±S.D.	t
Normal Animals							
No treatment	13	234	87.6± 7.5		284	105.8±5.8	
Saline	10	238	88.5± 9.3		295	108.8±7.6	
Alkaline Saline	10	234	87.6± 8.3		291	107.9±9.0	
ACTH‡	15	274	101.4±13.5	2.8†	339	119.3±8.5	3.2‡
Cortisone	15	240	88.9±12.8	0.9	311	112.7±9.7	1.1
Thyroxine	15	231	85.3± 8.6	0.4	318	114.9±6.2	2.2*
TSH	15	255	95.6± 8.3	2.6†	311	113.0±7.7	1.5
Hypophysectomy	4				202	60[4]	
Adrenalectomy	4				230	76[4]	
Splenectomy[5]							
Saline Controls	3	239	89.0		284	107.0	
ACTH	9	254	94.3±12.7	1.1	298	109.7±7.7	0.3
Thyroxine	9	242	90.9± 8.1	1.1	327	117.1±6.9	2.5*

[1] Clot dissolution time.
[2] Antifibrinolytic units.
[3] Comparisons are made with the injected controls. p values: * = <0.05, † = <0.02, ‡ = <0.01.
[4] Tested against standard trypsin after 30 minutes incubation only. The normal value with trypin—104 AFU/ml of plasma.
[5] Previous studies showed no changes in the antifibrinolytic titer after splenectomy.

In general, these findings are in agreement with the observations of Ungar and coworkers (1951) namely, an increased antifibrinolytic

activity after the administration of adrenocorticotropin to normal animals and no alteration in the plasmin inhibitor titer when administered to splenectomized animals. However, the results presented do not seem to substantiate the concept suggested by these workers that adrenocorticotropin affects the antifibrinolytic activity of normal plasma by accelerating the rate of combination between plasmin and plasmin inhibitor. Instead, the recorded data tend to show that the observed increased antifibrinolytic activity is a result of an actual rise in the plasmin inhibitor level in the blood.[4]

SUMMARY

The degree of combination between plasmin and plasmin inhibitor was found to depend on the incubation time and temperature of the reactants.

Administration of adrenocorticotropin produced a significant increase in the plasmin inhibitor titer of blood. The injection of thyrotropin or thyroxine caused elevations of the plasmin inhibitor in blood. Cortisone produced no change. The plasmin inhibitor titer of rat plasma 10 days after removal of the hypophysis or of the adrenals, was found to be greatly reduced. Splenectomy did not alter the plasmin inhibitor level of plasma. In splenectomized rats adrenocorticotropin administration did not produce any change in the plasmin inhibitor titer of the blood. On the other hand, administration of thyroxine to splenectomized rats was found to produce an elevation of the titer.

The significance of these findings has been discussed.

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[4] It may be pointed out that the observed increase in antiplasmin activity can also be caused by a decrease in the plasmin of the blood. The test, as employed, does not differentiate between fibrinolytic and antifibrinolytic activity.

NOTES AND COMMENTS

THE INFLUENCE OF CORTISONE ON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID*

Several groups of workers have found a depression of thyroid I^{131} uptake in cortisone-treated rats (Money *et al.*, 1951, Verzár and Vidovič, 1951, Perry, 1951, Albert *et al.*, 1952). An inhibition of the pituitary thyrotrophic mechanism was ruled out as the cause of this phenomenon by the findings that the rate of release of radioiodine from the thyroid is not reduced by cortisone (Perry, 1951, Albert *et al.*, 1952) and that the morphology of the thyroid in cortisone-injected rats suggests a compensatory hypersecretion rather than a diminished output of thyrotrophin (TSH) (Halmi and Barker, 1952). Albert *et al.* (1952), in a trenchant analysis of the cortisone-induced changes in thyroid function, pointed out that reduced I^{131} uptake may be the result of various disturbances, such as an increased renal elimination of iodide, an augmentation of the iodide space or an inhibition of the concentration of iodide by the thyroid. One might add to this list an interference with the organic binding of iodine in the thyroid.

The present study was undertaken to ascertain the effect of large doses of cortisone on the ability of the thyroid to concentrate iodide. Since Woodbury *et al.* (1951) reported that exogenous TSH is less effective in raising the thyroidal I^{131} uptake of hypophysectomized rats if cortisone is administered simultaneously, we also investigated the influence of cortisone on the thyroid iodide trap of hypophysectomized rats receiving TSH. Finally we examined the effect of cortisone on the rise in the thyroid: serum iodide ratio which follows the administration of goitrogens (Vanderlaan and Vanderlana, 1947).

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain were used in this study. In the first experiment 10 rats were injected with daily doses of 8 mg of cortisone[1] for 10 days. Ten uninjected control rats were pair-fed. In the second experiment hypophysectomized rats (100–150 gm.) were obtained 8 days after operation from the Hormone Assay Laboratories, Chicago and divided into 3 groups. The first group received no treatment, animals in the second group were injected with 1 mg. of TSH (USP Thyrotropin Reference Substance) daily for 10 days, while to those of the third group 1 mg. of TSH and 5 mg. of cortisone were administered daily over a period of 10 days. In the third experiment 10 rats received 10 daily injections of 10 mg. of propylthiouracil in an alkaline solution, while 10 pair-fed rats were given 10 mg. of cortisone daily for 10 days in addition to the same amount of propylthiouracil. All unoperated rats received powdered Rockland chow, while the hypophysectomized animals were given the same diet in pellet form and supplemented with horse meat and oranges.

The iodide concentrating ability of the thyroids was determined by the method of Vanderlaan and Greer (1950). All rats were injected subcutaneously with 5 mg. of propylthiouracil in an alkaline solution. Forty-five minutes later they received an injection of 5 μc. of carrier-free radioiodide.[2] One or two hours following the latter injection the rats were anes-

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[1] Cortisone (Cortone acetate Merck) was generously supplied by Dr. E. Alpert, Merck and Co., Rahway, N. J.

[2] Radioiodide was obtained through the courtesy of Dr. T. C. Evans, Radiation Research Laboratory, State University of Iowa.

thetized with ether and blood was withdrawn from the abdominal aorta. The thyroids were dissected out, weighed on a torsion balance and squashed on a piece of filter paper, which was placed on an aluminum planchet and covered with scotch tape. One tenth of a ml. of serum was pipetted onto a piece of filter paper of the same size, which then was also fixed to a planchet with scotch tape. The radioactivity of the thyroids and the blood samples was determined by means of a thin mica-window Geiger-Müller counter. Since the propylthiouracil almost completely prevents the organic binding of the subsequently administered I^{131} (Vanderlaan and Vanderlaan, 1947), the radioactivity of the thyroid can be regarded as the measure of radioiodide collection by the gland. The thyroid:serum iodide ratio is expressed as the quotient of the respective activities of 100 mg. of thyroid tissue and 0.1 ml. of serum.

RESULTS

Table 1 shows that the thyroid:serum iodide ratio in rats treated with 8 mg. of cortisone daily for 10 days was somewhat higher than that of pair-fed controls. Table 2 summarizes the observations on groups of hypophysectomized rats which received a) no treatment, b) 1 mg. of TSH daily for 10 days, and c) 1 mg. of TSH and 5 mg. of cortisone daily for 10 days. The low thyroid:serum iodide ratio found in the untreated hypophysectomized rats is in agreement with the findings of Greer (1949). TSH markedly elevated the thyroid:serum iodide gradient and cortisone did not exert any significant influence on this response to TSH. The results of the third experiment, which are shown in Table 3,

TABLE 1. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS GIVEN 8 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF THEIR PAIR-FED CONTROLS

Group	No. of rats	Average		Thyroid: serum iodide ratio (mean ± st. error)
		Starting weight	Final weight	
		gm.		
Cortisone	10	194	153	27.9 ± 2.6 ⎫ P = 0.02
Control	10	210	213	20.8 ± 0.4 ⎭

TABLE 2. THYROIDAL IODIDE CONCENTRATING ABILITY OF GROUPS OF HYPOPHYSECTOMIZED RATS RECEIVING NO TREATMENT, 1 MG. OF TSH DAILY FOR 10 DAYS OR 1 MG. OF TSH + 5 MG. OF CORTISONE DAILY FOR 10 DAYS

	No treatment	TSH only	TSH + cortisone
No. of rats	14	13	13
Thyroid: serum iodide ratio (mean ± st. error)	5.0 ± 0.7	32.9 ± 2.6	30.2 ± 2.6
		P = 0.4	

TABLE 3. THYROIDAL IODIDE CONCENTRATING ABILITY OF RATS RECEIVING 10 MG. OF PROPYLTHIOURACIL + 10 MG. OF CORTISONE DAILY FOR 10 DAYS AND OF PAIR-FED CONTROLS GIVEN 10 MG. OF PROPYLTHIOURACIL DAILY FOR 10 DAYS

Group	No. of rats	Average		Thyroid: serum iodide ratio (mean ± st. error)
		Starting weight	Final weight	
		gm.		
Propylthiouracil + cortisone	10	195	152	92.7 ± 15.7 ⎫ P = 0.7
Propylthiouracil only	10	199	193	101.8 ± 6.4 ⎭

indicate that the simultaneous administration of 10 mg. of cortisone and of 10 mg. of propylthiouracil daily failed to prevent the rise in the thyroid:serum iodide ratio which was elicited by 10 daily doses of 10 mg. of the latter drug alone.

DISCUSSION

The results presented in this paper indicate a) that the defective thyroid I^{131} uptake observed by others in cortisone-treated rats is not likely to have been the sequel of an interference of this steroid with the ability of the thyroid to concentrate iodide, b) that the partial inhibition exerted by cortisone upon the TSH-induced rise in thyroid I^{131} uptake of hypophysectomized rats (Woodbury *et al.*, 1951) is equally unlikely to have been the result of interference with the iodide trapping mechanism, and c) that, indeed, a striking elevation of the thyroid: serum iodide ratio in response to a goitrogen is compatible with the simultaneous injections of large amounts of cortisone. The fact that cortisone-treated rats had an apparently slightly higher thyroid:serum iodide ratio than that of their pair-fed controls is in agreement with the concept that this steroid is not a TSH inhibitor, since the thyroid:serum iodide gradient drops if TSH does not act on the thyroid. Furthermore, it harmonizes with histological findings indicating an increased thyrotrophic stimulation of the thyroids of cortisone-treated rats (Halmi and Barker, 1952). Since both the pituitary thyrotrophic mechanism and the thyroid iodide trap thus appear to be ruled out as points of attack of cortisone, the following hypotheses remain to be verified or refuted: a) cortisone affects the extrathyroidal metabolism of iodide (renal excretion, iodide space), b) it partially inhibits a phase of the organic binding of iodine in the thyroid.

SUMMARY

Cortisone did not materially affect the iodide trapping ability of the thyroids of normal rats, of hypophysectomized rats given thyrotrophin, or of rats chronically treated with propylthiouracil.

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PROGESTERONE IN THE SYSTEMIC BLOOD OF WOMEN AND MONKEYS DURING THE PUERPERIUM[1]

The unexpected detection of progesterone in systemic blood samples drawn from two women and three monkeys at intervals for several days after the birth of their offspring (Forbes, 1951) suggested the desirability of attempting to learn more about progesterone levels existing during the time when lactation may be initiated. Blood specimens were therefore obtained from additional mothers during the postpartum period. The following account summarizes a study of specimens from a total of three monkeys and eleven women.

As in previous investigations, levels of "free" and "bound" progesterone in plasma were determined according to a standard procedure, employing a bioassay believed to be sensitive, accurate, and specific at least to the extent that other naturally occurring compounds tested have not duplicated the cytological response on which the assay is based (Hooker and Forbes, 1947, 1949a). The production by estrogen of false negative responses to the assay has been reported (Salhanick, Olsen, and Hisaw, 1951); a subsequent study (Zarrow and Neher, 1951) indicates that what appear to be physiological concentrations of estrogen do not cause false negative responses.

Blood samples of about 1 cc. each were drawn from arm veins of the women and leg veins of the monkeys. The initial specimens were taken from the three monkeys ¾, 4 and 30 hours after delivery. Thereafter blood was obtained every two to five days for 25–36 days. Specimens were secured, usually daily, for four to ten days (the duration of hospital stay) from the human donors, the initial sample being drawn within 12 hours after delivery. All human samples were obtained between 10:00 A.M. and 3:15 P.M. A total of 105 blood samples was assayed for free progesterone.

The ether- and acetone-insoluble fractions of occasional plasma samples were also assayed for bound progesterone. The concentration of the latter never exceeded 0.3 μg./cc. plasma; frequently, no progesterone could be detected.

One of the monkeys (B3; see graphs) nursed one of her twin babies; no lactation record was available for the other two monkey mothers. Six of the women (H7, 9, 12, 10, 15, 11) nursed their babies. Three other human mothers did not try to do so; of these, two (H16 and H18) definitely lactated, while the mammary glands of the third mother (H13) became swollen, hard, and tender soon after delivery. No signs of actual or potential milk production were reported for the remaining two women (H17 and H14).

Inspection of the graphs of progesterone levels in relation to time has not revealed a correlation between the shape of the curve and the presence or absence of lactation. Indeed, similar curves, for example, exist for H9 (nursed baby) and H16 (lactated, did not nurse) and for H18 (lactated, did not nurse) and H17 (apparently did not lactate). Although progesterone could not be detected in every blood sample, it was present in low concentrations in at least some of the samples from each donor. Progeseterone was found as long as 36 days after parturition in monkeys and 10 days postpartum in women.

This experiment would seem to shed no light on the relation, if any, of postpartum blood levels of systemic progesterone to the initiation of lactation. It is surprising that

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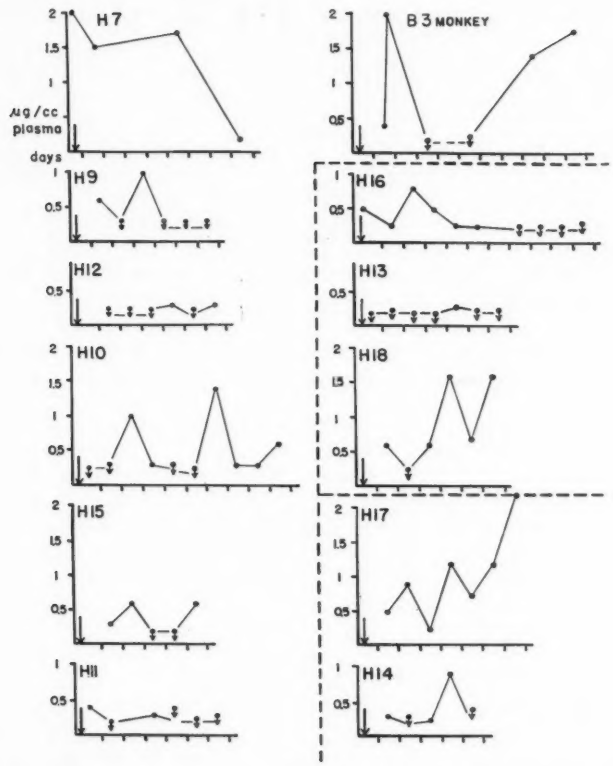

Assay results in μg. progesterone/cc. plasma are plotted during the puerperium. The time of delivery is indicated by the large arrow close to the ordinate. The dot and arrow symbol means that less than the indicated concentration of progesterone was present. Donors H7, 9, 12, 10, 15, 11 and B3 nursed their babies. H13 probably, H16 and H18 definitely, lactated but did not nurse. H17 and H14 did not lactate.

the hormone continues to be present after separation of the placenta. Neher and Zarrow have reported (1950) that 2 μg. progesterone/cc. serum were detected 10 days postpartum in the ewe.

SUMMARY

In three monkeys and eleven women, relatively small concentrations of progesterone were present in the systemic blood for several days after delivery of the offspring. No correlation was apparent between observed progesterone levels and the lactation periods.

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THE USE OF COLLODION ULTRAFILTRATES OF HUMAN URINE FOR STEROID DETERMINATIONS

INTRODUCTION

The practicability of determining the content of more than one hormone in a single sample of human urine is limited, essentially, by the concentration of the desired hormones in the urine. Thus, the gonadotrophins, pregnanediol and the adrenal cortical steroids are usually present in such small amounts, particularly in non-pregnant subjects, that quantities of urine varying from about $\frac{1}{5}$ of a day's output to as much as a 48 hour output, or more, may be required for the determination of only one of these. It is often desirable, however, from the standpoint of the patient's convenience, as well as the research objective, to know the excretion level of the gonadotrophins and of a steroid hormone or its metabolites during a particularly limited period of time, such as 24 or 12 hours. Our use of the Gorbman (1945) method for extracting gonadotrophins by ultrafiltration through a collodion membrane suggested that a simple solution to this problem might be at hand if the ultrafiltrate could be used for the steroid assays. This has been tested by determination of the 17-ketosteroid and pregnanediol content of several urines before and after filtration through collodion. We have found that, with certain reservations, the technique is a practical one, and, to our knowledge, has not been reported before.

METHODS AND MATERIALS

A total of 39 complete 24 hour urines were studied for total 17-ketosteroid, and 26 urines were studied for pregnanediol content before and after ultrafiltration. The urines were collected from a group of young healthy female inmates of a home for the feeble minded. All urines were kept refrigerated, with thymol as the only preservative, until used.

Gonadotrophins were extracted on aliquots of each urine, as described by Gorbman (1945). In the pregnanediol series, a single aliquot of urine, equal to $\frac{1}{5}$ of the day's output, was first removed, the remaining $\frac{4}{5}$ of the day's urine ultrafiltered, and pregnanediol determined on a single $\frac{1}{5}$ aliquot of the ultrafiltrate, and on the aliquot of unfiltered urine. For the 17-ketosteroid series, a 10.0 cc. volume of unfiltered urine was first removed, the remainder of the urine ultrafiltered, and the 17-ketosteroid determinations carried out on a 10.0 cc. volume of the ultrafiltrate and the 10.0 cc. aliquot of unfiltered urine. The method of Sommerville, Gough and Marrian (1948) was used for pregnanediol, and that of Drekter, *et al.* (1947) for 17-ketosteroid analyses.

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TABLE 1. TOTAL 17 KETOSTEROID CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values are presented in three groups: (A) urines containing less than 10.0 mg./24 hours; (B) urines containing between 10.1 and 15.0 mg./24 hours; and (C) urines containing more than 15.0 mg./24 hours. The values shown in the first two columns of each group are mg. of 17-ketosteroid per 24 hours. The group means for the percentage change from the value of the urine before filtration are derived from the difference between the before and after filtration group means, rather than from the sum of the individual percentages. There is a slight discrepancy between these two values only in the case of the first group of urines (less than 10.0 mg./24 hours).

A. Less than 10.0 mg. per 24 hours			B. 10.1 to 15.0 mg. per 24 hours			C. More than 15.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
4.9	5.8	+18	10.1	7.8	−23	15.1	13.9	−7.9
5.0	6.3	+26	10.8	8.0	−26	15.8	14.6	−7.6
6.3	5.2	−17	10.9	8.3	−24	16.0	16.0	0
6.4	9.5	+48	11.2	9.7	−13	16.0	16.0	0
6.6	8.0	+21	11.4	9.5	−17	16.6	15.3	−7.8
6.9	6.0	−13	12.6	12.6	0	17.3	14.7	−15
7.2	7.9	+9.7	13.1	13.1	0	17.5	17.0	−2.9
7.3	4.8	−34	13.2	10.5	−20	20.4	19.9	−2.4
9.2	7.0	−24	13.4	15.3	+14	20.9	14.4	−31
9.4	11.4	+21	15.0	15.0	0	21.6	19.6	−9.3
9.5	8.0	−16				21.9	21.9	0
9.8	7.2	−27				22.0	21.2	−3.6
9.9	6.6	−33				22.6	22.6	0
						25.5	25.2	−1.2
						26.5	21.4	−19
						48.6	50.3	+3.5
Group means:								
7.6	7.2	−5.3	12.2	11.0	−9.9	21.5	20.2	−6.1
Mean of % change, regardless of sign:		23.7			13.7			6.9

* From the value of the urine before filtration.

RESULTS

17-ketosteroids. The 17-ketosteroid values, in terms of mgs./24 hours, are shown in Table I. The values are arranged in three groups, according to the quantity of steroid excreted per day, in order to show its relation to the percentage change (expressed in all cases in relation to the amount present in the urine before filtration) following filtration. There was a very obvious inverse relation between the daily level of 17-ketosteroid excretion and the amount of variation in the values of the percentage change following ultrafiltration. This is shown by the decrease in the value of the mean of the percentage change, *calculated without regard to the sign of this change* (shown at the bottom of each group of values). The true means showed little difference between the three groups of urines, and the fact that most of the minus changes were found in the intermediate and high content urines is an indication of a probable slight but real loss of steroid as a result of filtration.

Pregnanediol. Not as many urines were examined for pregnanediol content, and the values, though extending over as wide a relative range as did the 17-ketosteroids, did not compare with the latter as far as absolute amounts are concerned. Nevertheless, very much the same sort of relationship was seen (Table 2), and what has been said about the

TABLE 2. PREGNANEDIOL CONTENT OF HUMAN URINE BEFORE AND AFTER ULTRAFILTRATION THROUGH COLLODION

The values presented are in two groups: (A) urines containing less than 1.0 mg./24 hours; and (B) urines containing more than 1.0 mg./24 hours. As in Table 1, the values in the first two columns of each group are mg. of pregnanediol per 24 hours, and the group means for the per cent change are derived from the difference between the before and after filtration group means.

A. Less than 1.0 mg. per 24 hours			B. More than 1.0 mg. per 24 hours		
Before Filtration	After Filtration	% change*	Before Filtration	After Filtration	% change*
0.17	0.25	+47	1.17	0.90	−23
0.19	0.09	−52	1.35	0.44	−67
0.20	0.20	0	1.37	1.19	−13
0.25	0.20	−20	1.38	1.47	+6.5
0.26	0.28	+7.7	1.62	1.41	−13
0.26	0.22	−15	1.70	1.48	−13
0.28	0.23	−18	1.70	1.77	+4.1
0.28	0.39	+39	1.77	1.55	−12
0.36	0.58	+61	1.89	1.68	−11
0.40	0.25	−37	1.99	1.90	−4.5
0.74	0.55	−26	2.07	2.34	+13
0.86	0.13	−85	2.42	2.23	−7.9
0.96	0.21	−78	4.90	7.50	+53
Group means:					
0.40	0.27	−32	1.95	1.97	−1.03
Mean of % change, regardless of sign		37%			19%

* From the value of the urine before filtration.

size of the change following filtration and the amount of 17-ketosteroid in the urine, applies in general as well to the pregnanediol series.

DISCUSSION

When one considers that the two values compared in this study were obtained from single aliquots of urine, the amount of variation between them is not surprising and should not be disturbing. For clinical purposes there can hardly be any question of the value of using the ultrafiltrate of urine for a steroid determination, since the alternative of using separate 24 hour collections will be associated with variations at least as large as those resulting from the passage through collodion. In addition, the importance of such determinations for clinical purposes is not obviated by a loss or gain from the real value of roughly 20%. However, the difference from the true value as a result of filtration may be serious in certain areas of research where relatively small differences in output are anticipated, but this is to some extent minimized by the fact that the percentage change is more frequently a large one almost only when one is dealing with relatively small concentrations of steroid. If this is recognized, and data interpreted accordingly, there is no reason why the method cannot be used, especially if the determinations are made on at least duplicate aliquots of the ultrafiltrate. It has thus been of inestimable help in allowing us to perform a single experiment to determine simultaneously the effect of progesterone on gonadotrophin output, and the conversion of progesterone to pregnanediol in relation to the phases of the menstrual cycle.

SUMMARY

The 17-ketosteroid and pregnanediol content of human urine after ultrafiltration through collodion is shown to vary from the content of these steroids in the unfiltered urine within limits small enough to allow the ultrafiltrate to be used for the determination of excretion levels of these substances.

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THE 1953 ANNUAL MEETING

The Thirty-fifth Annual Meeting of The Endocrine Society will be held in the Hotel Statler, New York, New York, Thursday, Friday, and Saturday, May 28–29–30, 1953.

The Committee on Local Arrangements is Dr. Earl T. Engle, Chairman, with Drs. Rulon W. Rawson and Sidney C. Werner as members of the Committee.

All Scientific Sessions will be held in the Hotel Statler. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, May 29, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Reservations should be made directly with the Hotel Statler, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 1, 1953.

THE AMERICAN GOITER ASSOCIATION

1953 ANNUAL MEETING

The 1953 Annual Meeting will be held May 7, 8 and 9, 1953, in the Drake Hotel, Chicago, Illinois. The program for the three day meeting will consist of papers and discussions dealing with goiter and other diseases of the thyroid gland.